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CONTENTS OF VOLUME 33

NO. 1, JULY, 1943

BURDICK, H. O., HUBER WATSON, VINCENT CIAMPA AND THOMAS CIAMPA
A Rapid Test for Pregnancy Gonadotropins on the Basis of Induced Ovulations in Mice 1

WEICHERT, CHARLES K., AND HENRY B. HALE
Inhibition of Estrogenic Effects on the Reproductive System of the Male Rat by Testosterone Injections 16

DAVIS, BERNARD D., AND BENJAMIN F. JONES
Beneficial Effect of Estrogens on Altitude Tolerance of Rats 23

KRICHESKY, B., J. A. BENJAMIN AND B. ROSENBERG
Effect of Gonadotropic Hormones on Intraocular Prostatic Implants in the Male Rabbit 32

HISAW, FREDERICK L.
Androgens and Experimental Menstruation in the Monkey (Macaca mulatta) 39

PUGSLEY, L. I., AND C. A. MORRELL
Variables Affecting the Biological Assay of Estrogens 48

NEW BOOKS 62

NO. 2, AUGUST, 1943

TRENTIN, J. J., A. A. LEWIS, A. J. BERGMAN AND C. W. TURNER
Nature of the pituitary factor stimulating mammary duct growth 67

DEY, F. L.
Evidence of hypothalamic control of hypophyseal gonadotropic functions in the female guinea pig 75

WHICHER, C. H., AND E. M. WATSON
Effects of thyrotropic hormone, gonadotropic factor, pituitary growth substance and insulin upon the phosphatase content of rat femurs 83

SAMUELS, LEO T., ROGER M. REINECKE AND KENNETH L. BAUMAN
Growth and metabolism of young hypophysectomized rats fed by stomach tube 87

HIMWICH, HAROLD E., JOSEPH F. FAZEKAS AND EDMUND HOMBURGER
Effect of hypoglycemia and anoxia on the survival period of infant and adult rats and cats 96

MARX, WALTER, MIRIAM E. SIMPSON, CHOH HAO LI AND HERBERT M. EVANS
Antagonism of pituitary adrenocorticotropic hormone to growth hormone in hypophysectomized rats 102

RICE, KATHERINE K., AND CURT P. RICHTER
Increased sodium chloride and water intake of normal rats treated with desoxycorticosterone acetate 106

NOTES AND COMMENTS

FISCHER, E.
Preparation of an active fraction of the pineal gland 116

NEW BOOKS 118

ASSOCIATION NOTICE 120

NO. 3, SEPTEMBER, 1943

YOUNG, W. C., AND ROBERT M. YERKES
Factors Influencing the Reproductive Cycle in the Chimpanzee; the Period of Adolescent Sterility and Related Problems 121

LEBLOND, C. P., AND J. GROSS
Effect of Thyroidectomy on Resistance to Low Environmental Temperature 155

JOSEPH, SAMUEL, MALVINA SCHWEIZER AND ROBERT GAUNT
Studies on the Response of Hypophysectomized Rats to Intraperitoneal Glucose Injections 161

WARWICK, E. J., AND L. E. CASIDA
Effects of Pituitary Gonadotropins on Estrual Phenomena in Ewes 169

BERGMAN, H. C., AND DAVID KLEIN
Relation of Body Weight to Liver Glycogen Storage Potency of Adrenal Cortical Extracts 174

MATTHEWS, CHARLES S., FREDERICK E. EMERY AND PAUL L. WEYGANDT
Effect of Continued Oral Administration of Diethylstilbestrol on Blood Pressure, Heart Rate and Respiration of Albino Rats 177

INGLE, DWIGHT J., AND JAMES NEZAMIS
Further Study of the Specificity of the Diabetogenic Effect of Diethylstilbestol in the Partially Depancreatized Rat 181
NOTES AND COMMENTS
HELLER, CARL G., ALBERT SEGALOFF AND WARREN O. NELSON
Effect of Testosterone Propionate on the Pituitary Gonadotropic Potency of the Castrated Male Rat 186

NO. 4, OCTOBER, 1943

NATHANSON, IRA T., AND HILDEGARD WILSON
Factors Affecting Colorimetric Urinary 17-Ketosteroid Determinations ... 189
ZONDEK, BERNHARD, AND FELIX SULMAN
Inactivation of Estrone and Diethylstilbestrol by Microorganisms 204
SEGALOFF, ALBERT
The Intrasplenic Injection of Estrogens and their Esters 209
HOLLANDER, FRANKLIN, BRUNO KRISS, EMANUEL KLEMPNER AND ROBERT T. FRANK
Constancy of Androgen Concentration in the Urine 217
DOSNE, CHRISTIANE
Study of Interrelationship of Pancreatic Diabetes with Endocrine Glands in the Toad 224
ENGSTROM, WILLIAM W., AND HAROLD L. MASON
A Study of the Colorimetric Assay of Urinary 17-Ketosteroids 229
EVANS, HERBERT M., MIRIAM E. SIMPSON AND CHOH HAO LI
Inhibiting Effect of Adrenocorticotropic Hormone on the Growth of Male Rats 237
ZEINER, FREDERICK N.
Studies on the Maintenance of Pregnancy in the White Rat 239
KIBLER, H. H., A. J. BERGMAN AND C. W. TURNER
Relation of Certain Endocrine Glands to Body Weight in Growing and Mature New Zealand White Rabbits 250
NOTES AND COMMENTS
SALMON, A. A., AND E. C. HAMBLEN
Recovery of Injected Chorionic Gonadotropin from Rabbit Urine 257
NEW BOOKS 260

NO. 5, NOVEMBER, 1943

SIMPSON, MIRIAM E., HERBERT M. EVANS AND CHOH HAO LI
Bioassay of Adrenocorticotropic Hormone 261
MCSHAN, W. H., HAROLD R. WOLFE AND ROLAND K. MEYER
Factors Affecting the Action of Antigonadotropic Sera in Immature Rats .. 269
ROOS, A.
Assay of Adrenal Cortical Extracts in Adrenalectomized Rats Exposed to Cold 276
HANSEN, LORENZ P., A. CANTAROW, A. E. RAKOFF AND K. E. PASCHKIS
Studies of the Zimmermann Reaction: Factors Affecting the Color Intensity; Relation of Molecular Structure to Color Production 282
CLEVELAND, RUCKER, SUSAN WILKES AND GRACE SABOTKA
Color Measurement of the Sexual Skin of the Macacus Rhesus by the Munsell System of Color Annotation 289
GOLDNER, M. G., AND G. GOMORI
Alloxan Diabetes in the Dog 297
CANTAROW, A., K. E. PASCHKIS, A. E. RAKOFF AND L. P. HANSEN
Studies on Inactivation of Estradiol by the Liver 309
GAEBLER, O. H., AND S. M. TARNOWSKI
Effects of Estrone, Ascorbic Acid and Testosterone Propionate on Nitrogen Storage and Insulin Requirement in Dogs 317
NELSON, WARREN O., ROBERT GAUNT AND MALVINA SCHWEIZER
Effects of Adrenal Cortical Compounds on Lactation 325

NO. 6. DECEMBER, 1943

ZONDEK, BERNHARD, FELIX SULMAN AND JOACHIM SKLOW
Inactivation of Stilbestrol by Liver in Vitro 333
KOSTER, RUDOLF
Hormone Factors in Male Behavior of the Female Rat 337
RICHTER, CURT P., AND SYLVIA HELFRICK
Decreased Phosphorus Appetite of Parathyroidectomized Rats 349

WILSON, JAMES G., AND HARRIET C. WILSON
Reproductive Capacity in Adult Male Rats Treated Prepuberally with Androgenic Hormone 353
LOWENSTEIN, BERTRAND E., AND RAYMUND L. ZWEMER
Resistance of Rats to Potassium Poisoning after Administration of Thyroid or of Desoxycorticosterone Acetate 361
GORDON, ALBERT S., FRANK J. TORNETTA, SAVINO A. D'ANGELO AND HARRY A. CHARIPPER
Effects of Low Atmospheric Pressures on the Activity of the Thyroid, Reproductive System and Anterior Lobe of the Pituitary in the Rat 366
DEMPSEY, EDWARD W., AND DAVID L. BASSETT
Observations on the Fluorescence, Birefrigence and Histochemistry of the Rat Ovary during the Reproductive Cycle 384
NOTES AND COMMENTS
SCHILLER, SARA, AND RALPH I. DORFMAN
Influence of Urinary Cortin-like Material on Water Intoxication in the Adrenalectomized Rat 402
CORNER, GEORGE W., SR.
-Tropic versus -Trophic in the Terminology of the Pituitary Hormones 405
NEW BOOKS 409
INDEX TO VOLUME 33 413

ENDOCRINOLOGY

VOLUME 33 JULY, 1943 NUMBER 1

A RAPID TEST FOR PREGNANCY GONADOTROPINS ON THE BASIS OF INDUCED OVULATION IN MICE

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EVER since Smith and Engle (1927) showed that ovulation could be induced in mice by transplants of pituitaries, numerous investigators have reported this response in several species of animals following injections of various gonadotropins. Practically all of the observations on the induction of ovulation in rats and mice, however, have been made following a series of injections over a period of days, although Cole and his co-workers (Cole, *et al.*, 1932; Saunders and Cole, 1936; Cole, 1936, 1937, 1940) caused ovulation in late immature rats by single injections of an extract of pregnant mare serum. But this ovarian response was delayed until 72 or more hours after the injection. Subsequently, Burdick and Whitney (1941) reported that a single subcutaneous injection of human chorionic gonadatropin caused ovulation in the non-pregnant adult mouse within 18 hours after the injection. Similar injections of untreated human pregnancy urine also induced ovulations in pregnant and late immature mice.

In view of the fact that no quantitative studies have been made on this rapid response of the mouse ovary to pregnancy gonadotropins it was decided to determine the minimal ovulating dose of human chorionic gonadotropin in mature diestrous and pregnant mice. It also seemed desirable to compare the ovulating capacity of human chorionic gonadotropin with that of pregnant mares' serum when injected subcutaneously into the late immature mouse. Furthermore, the possible use of the mouse in a rapid test for pregnancy was considered.

PROCEDURE

Albino mice of the Rockland strain were used throughout these experiments. The mature non-pregnant females were selected by the vaginal smear method and were usually injected late in the first 24-hour period of diestrus.

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It will be shown later that induced ovulations under such conditions need not be confused with normal cyclic ovulations. Pregnant mice were timed from the appearance of vaginal plugs, or selected in the second half of pregnancy by their evident increase in size and weight. Only those animals in which the embryos had become implanted were considered in this report. The immature animals varied in age from 27 to 47 days and in weight from 7 to 19.5 gm. All immature animals included in this report had intact vaginal orifices at the time of autopsy.

Each animal received only one subcutaneous injection of the gonadotropin and was killed 14 to 26 hours later. In a few instances, for special reasons, the mice were not examined until 40 to 50 hours after the injection. Each genital tract was removed from the body cavity and the tubal ampullae examined under a binocular dissecting microscope. A distended ampulla indicated ovulation. For counting the eggs enclosed in each ampulla the tracts were prepared by the method outlined in an earlier report (Burdick and Whitney, 1941).

No attempt was made to compare the action of various batches of the same gonadotropin. Only one sample each of pregnancy urine extract, no. 77279, and pregnant mares' serum, no. PK03, was tested.[1] Necessary dilutions were made with distilled water at the time of injection. This was done to prevent possible deterioration of the extract.

For the purposes of this investigation, a minimal ovulating dose is defined as the smallest amount of gonadotropin which, administered in a single subcutaneous injection, will induce ovulation in at least 50 per cent of the treated animals within 24 hours after the injection.

RESULTS

Minimal Dose of Chorionic Gonadotropin Necessary to Induce Ovulation in Mature Diestrous Mice

Over 40 animals, not indicated in the table, were used in a preliminary series of dosages ranging downward from 150 I.U. of the chorionic gonadotropin to determine the range of ovulating potency of this material. When it was found that a dose of 5 I.U. was apparently as effective as any of the higher dosages, more animals were used for single injections varying in amount from 3 to 0.75 I.U. with results as shown below and in table 1.

Results with 3 I.U. The 8 mice in this series all ovulated. The number of ova varied from 5 to 15 with an average of 9.8. Only 2 animals had fewer than 8 ova. A total of 79 ova was found.

Results with 1 I.U. All 11 animals used in this series ovulated. The number of ova ranged from 4 to 13 with a total of 104, an average of 9.4 ova each. Only one animal (mouse 73) had fewer than 8 ova. This animal was killed only 17 hours after being injected.

Results with 0.75 I.U. Only one of 7 animals in this group ovulated.

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[1] Grateful acknowledgment is made to E. R. Squibb & Sons, New Brunswick, N.J., for the chorionic gonadotropin (Follutein), and to Schering Corp., Bloomfield, N. J., for the pregnant mares' serum gonadotropin (Anteron).

There were but 4 ova in the ampullae. Evidently this amount is subliminal.

Effect of dosage on ovaries. It soon became apparent that the number of follicles rupturing after a single injection of chorionic gonadotropin was quite independent of the amount given, provided an optimal ovulating dose had been used. The average number of ruptured

TABLE 1. EFFECT OF SINGLE INJECTIONS OF CHORIONIC GONADOTROPIN IN DIESTROUS MICE

Animal Number	Ova Found in the Ampullae			
	Left	Right	Total	
		3 I.U.		
48	6	5	11	
49	6	5	11	
50	6	5	11	
75	6	7	13	
76	7	8	15	
77	4	4	8	
78	3	2	5	
79	4	1	5	
				Average for group, 9.8 ova
		1 I.U.		
45	4	6	10	
46	7	5	12	
47	5	3	8	
70	3	7	10	
71	6	4	10	
72	5	8	13	
73	3	1	4	(17 hr. lapse)
74	4	7	11	
114	1	6	7	
115	7	6	13	
116	1	5	6	
				Average for group, 9.4 ova
		0.75 I.U.		
106				
107				
109				
110				
111	1	3	4	
112				
113				

follicles was no greater with an injection of 10 to 50 I.U. than with 1 to 3 I.U. Whether or not there might have been a quantitative difference following the use of more carefully fractioned dosages in the region of the threshold was not determined.

Quite generally, the larger doses of chorionic gonadotropin resulted in excessive and rapid development of corpora lutea. In several cases of slightly delayed autopsies, corpora lutea were prominent and

beginning to mushroom from the surface of the ovary. Follicular stimulation was frequently seen following the smaller doses.

Ovulation Induced in Pregnant Mice by Chorionic Gonadotropin

Some of these animals were injected with dosages as high as 100 I.U. of chorionic gonadotropin during the course of exploratory experiments, but it was soon discovered that smaller amounts were as effective as the larger dosages. Those animals receiving the larger injections have not been included in this series, nor have those found not to be pregnant when killed. The accompanying table 2 shows the results in the animals grouped according to the amount of injected material. In each group the animals are listed according to the decreasing number of hours between injection and autopsy. The number of eggs found in the ampullae is shown in the next three columns while the last column indicates the number and approximate size of the embryos. Such a description as a '5 mm. bead' indicates an overall measurement of the uterine wall surrounding the embryo.

Results with 20 I.U. All 7 animals in this group ovulated. The number of eggs in each ranged from 2 to 12 with an average of 7 eggs each. Two animals examined 16 and 17 hours after receiving the gonadotropin had only 4 and 2 eggs. Ovulation may not have been completed at these hours. However, another animal, mouse 40, killed 22 hours after injection, had only 3 ova in the ampullae. Embryos varied from 6 to 13 in number and 10 to 18 mm. in length.

Results with 10 I.U. The 6 animals in this series had all ovulated 14 to 22 hours after injection. Each of the 2 animals killed at 14 hours had 6 or more ova. Unfortunately the eggs in one tract, although seen in the swollen ampulla, were not counted before the ampulla was accidentally ruptured and the eggs lost. The number of eggs in this series varied from 3 to 13 with an average of 8 eggs each (allowing for at least 2 eggs lost). Embryos varied from 5 to 10 in number and 3 mm. to 22 mm. in length.

Results with 5 I.U. This group presented much the same picture as the preceding series except that one mouse had not ovulated at 15 hours and another had only one ovum at 16 hours. This is the last group in which there was a short time interval between injection to autopsy. The number of ova present in each ranged from 0 to 11. There were 6 to 13 embryos, ranging from 4.5 to 18 mm. in length.

Results with 0.1 I.U. Only 2 of the 9 animals comprising this series failed to ovulate. The average number of ova for the 7 ovulating animals approximated 5 each but for all 9 mice the average was only 4. The number of embryos was rather uniform, varying only from 8 to 10.

Results with 0.8 I.U. All 6 animals ovulated with 1 to 9 ova, an average of 6 each. Seven to 10 embryos were found in each mouse.

TABLE 2. INDUCED OVULATIONS IN PREGNANT MICE BY SINGLE INJECTIONS OF CHORIONIC GONADOTROPIN

Animal Number	Hours Injection to Autopsy	Induced Ovulation			Number and Size of Implanted Embryos		
		Right	Left	Total No. of Eggs	no.	mm.	
			20 I.U.				
4	24	6	5	11	8	14	
3	23	6	1	7	13	12	
40	22	3	0	3	7	16	
2	21	8	4	12	11	10	
6	21	5	4	9	7	18	
37	17	3	1	4	8	12	
5	16	0	2	2	6	14	
			10 I.U.				
41	22	2	3	5	6	10	
10	18	6	7	13	10	7	
29	18	8	5	13	10	3	(beads)
9	16	2	1	3	10	6	
7	14	lost	6	6+	7	6.5	
33	14	5	1	6	5	22	
			5 I.U.				
11	19	2	0	2	12	10	
17	18	4	5	9	10	11	
13	23	1	6	7	6	4.5	
16	17	8	3	11	11	18	
8	16	1	0	1	13	5	(beads)
32	15	0	0	0	8	8	
			1 I.U.				
104	35	5	2	7	8	9	
112	18	4	5	9	9	17	
102	26	5	6	11	9	22	
83	25	0	0	0	8	14	
91	25	2	2	4	Bead-like embryos		
86	24	0	2	2	8	8	
94	24	5	1	6	8	9	
103	22	0	0	0	10	8	
105	20	3	3	6	9	3	
			0.8 I.U.				
109	27	4	4	8	8	20	
122	27	3	6	9	9	3	(beads)
111	26	5	3	8	8	18	
116	26	1	0	1	9	16.5	
117	26	0	2	2	7	15	
71	18	4	4	8	9	3	
			0.7 I.U.				
118	26	5	7	12	9	19	
114	25	5	6	11	11	19	
115	24	8	4	12	11	18	
			0.5 I.U.				
119	28	0	0	0	11	20	
120	27	0	1	1	10	18	
87	24	0	0	0	8	9	
96	24	0	0	0	12	14	
93	24	0	0	0	9	15	
70	19	0	0	0	Bead-like embryos		

Results with 0.7 I.U. There were only 3 pregnant animals in this group but they form an interesting series because they were all in late pregnancy, having embryos 18 mm. or more in length. The number of embryos was greater than the average, as was also the number of ova found in the ampullae. This amount of chorionic gonadotropin from this particular sample (no. 77279) approximates the minimal dosage which will regularly induce ovulation in at least 50 per cent of the treated animals. This will be seen after an examination of the next series.

Results with 0.5 I.U. There was only one egg in the entire series of 6 animals. Therefore it is evident that this amount of chorionic gonadotropin is subliminal for uniformly inducing ovulations in albino mice with implanted embryos.

General Results. It has been found of no particular value to delay autopsies under these conditions more than 24 hours after the injection. On the other hand, 14 to 16 hours was frequently too early for the follicles to rupture. A delay of 18 to 20 hours is probably sufficient for complete induced ovulation.

Ovulation Induced in Immature Mice by Pregnant Mares' Serum Gonadotropin

Examination of table 3 shows that 73 animals received subcutaneous injections of pregnant mares' serum gonadotropin, the doses ranging from 100 to 1 I.U. Four of 6 animals receiving 100 I.U. ovulated. It will be noted that all ovulations were in the group which weighed 15 to 18 gm. In the animals 27 to 47 days old, weighing less than 12 gm., no evidences of ovulation were found although the follicles were stimulated.

With 50 I.U. of pregnant mares' serum gonadotropin, 3 animals weighing less than 12 gm. failed to ovulate, although these animals were 31 days of age. Of the 4 animals weighing 13.6 to 15.8 gm., ranging in age from 38 to 45 days, 3 ovulated.

A dosage of 25 I.U. produced no ovulations in the group weighing 8.3 to 11.5 gm., although some of these animals were as old as 38 days. In the heavier group, ovulation was induced in 4 of 7 animals. Animal no. 169, killed after 19 hours had 11 ova in the tubes, indicating that ovulation can be obtained as early as 19 hours. On the other hand, no ova were found in mouse no. 103, 45 days of age, weighing 15.1 gm. There was no evidence that a greater number of follicles ruptured when necropsy was delayed longer than 24 hours.

Table 3 shows that all animals subjected to injections of 10 I.U. of pregnant mares' serum gonadotropin were 30 to 31 days of age. Of these, none weighing less than 12 gm. ovulated, even though mouse no. 129, 9.5 gm., was killed 49 hours after injection. Of 9 animals weighing 12.3 to 17.5 gm., 7 ovulated. Again, a delay of 49 hours was unnecessary as animal no. 128, weighing 12.5 gm. and killed at 49

hours, had 1 ovum without granulosa cells in the lower portion of the left tube, thus revealing that ovulation must have taken place about 30 hours previously. Animal no. 158, 18.4 gm., examined at 20 hours, had 32 ova, the largest number found in this investigation. Mouse

TABLE 3. OVULATIONS INDUCED IN IMMATURE MICE BY PREGNANT MARES' SERUM GONADOTROPIN

Weight of Animal, gm.	Time Lapse, hours	Age Variation, days	Animals Used, no.	Results
		100 I.U.		
Under 12 (10.2–11)	21–45	27–47	2	No ovulation
Over 12 (15–18)	19–25	35–47	4	4 ovulations (5–9 ova)
		50 I.U.		
Under 12 (7–8.5)	18–28	31	3	No ovulation
Over 12 (13.6–15.8)	19	38–45	4	3 ovulations (3–8 ova)
		25 I.U.		
Under 12 (8.3–11.5)	18–20	31–38	3	No ovulation
Over 12 (12.5–15.8)	19–43	31–45	7	4 ovulations (1–11 ova)
		10 I.U.		
Under 12 (9.5–10.5)	26–49	30–31	3	No ovulation
Over 12 (12.3–18.4)	21–49	30–31	9	7 ovulations (1–32 ova)
		5 I.U.		
Under 12 (8.5–11.5)	26–28	30–31	7	2 ovulations (1–2 ova)
Over 12 (12.3–17.5)	24–49	30–36	8	3 ovulations (7–17 ova)
		2.5 I.U.		
Under 12 (11.5–11.8)	27	30–31	2	No ovulation
Over 12 (13.5–15.5)	25–45	30–31	6	2 ovulations (6–18 ova)
		2 I.U.		
Under 12 (11.7)	24	34	1	No ovulation
Over 12 (12–15.2)	23–25	34	4	No ovulation
		1.5 I.U.		
Under 12 (10.8–11.5)	22–26	34	2	No ovulation
Over 12 (14–14.8)	22–25	34	3	No ovulation
		1 I.U.		
Over 12 (13.1–14)	22–26	34	5	No ovulation

no. 163, 16.2 gm., showed 22 ova after 23 hours. Both of these superovulations occurred in mice that were heavier than most of the animals used throughout this experiment.

With 5 I.U. some ovulations were obtained in animals weighing less than 12 gm. as well as in those weighing more. All 7 animals, weighing

TABLE 4. OVULATIONS INDUCED IN IMMATURE MICE BY CHORIONIC GONADOTROPIN

Weight of Animal, gm.	Time Lapse, hours	Age Variation, days	Animals Used, no.	Results
		50 I.U.		
Under 12 (11.5)	25	39	1	No ovulation
Over 12 (13–17)	19–44	39–45	4	2 ovulations (1–5 ova)
		30 I.U.		
Over 12 (14.4–15.7)	19–44	45	2	No ovulation
		20 I.U.		
Under 12 (11)	24	37	1	No ovulation
Over 12 (13–14.8)	23	36–37	2	No ovulation
		15 I.U.		
Over 12 (12–13.6)	27–40	30–31	3	3 ovulations (1–6 ova)
		10 I.U.		
Under 12 (6–10.5)	27–28	30–31	5	No ovulation
Over 12 (12.5–18.9)	21–50	30–36	12	5 ovulations (1–4 ova)
		5 I.U.		
Over 12 (13.5–19.5)	22–43	30–31	15	13 ovulations (1–10 ova)
		2.5 I.U.		
Over 12 (14.7–17.5)	22–26	31	5	4 ovulations (5–10 ova)
		2 I.U.		
Over 12 (12.8–14.8)	21–22	31	5	1 ovulation (1 ovum) Vesicular follicles
		1.5 I.U.		
Over 12 (12–14.7)	22–26	31	4	1 ovulation (2 ova) Vesicular follicles
		1 I.U.		
Over 12 (13.2–14.7)	27	31	5	No ovulation; some vesicular follicles

8.5 to 11.5 gm. and 30 to 31 days of age, showed considerable follicular stimulation, and 2 mice ovulated with 1 and 2 ova, respectively. In the heavier group, 3 of 8 animals ovulated. Mouse no. 125, weighing 15 gm., killed at 49 hours, had 7 ova which were without granulosa cells, indicating again that ovulation had taken place at least 24 hours earlier.

No animals under 12 gm. responded to 2.5 I.U. but 2 mice weighing over 12 gm. did ovulate. Mouse no. 169 weighing 15.3 gm., and killed at 26 hours, had 18 ova in the tubes, indicating that this small dose may be effective in producing a large complement of ova in some

animals. All of the heavy animals showed follicular stimulation in response to the injections.

In an effort to ascertain the minimal ovulating dose for this extract, a series of animals was injected with 2, 1.5 and 1 I.U. There were no induced ovulations in any of these groups, indicating that these doses were subliminal. It does not seem possible to suggest a minimal ovulation dose of pregnant mares' serum gonadotropin until a larger series of animals is examined. It will probably be found close to the 2.5 I.U. level for mice under these conditions.

Ovulation Induced in Immature Mice by Chorionic Gonadotropin

Sixty-six experimental animals having intact vaginal orifices were injected with various doses of chorionic gonadotropin. Injections of 100 to 200 I.U. were toxic and 6 of the animals died before the time of autopsy. In table 4 it will be seen that of the 5 animals injected with 50 I.U. of chorionic gonadotropin, only 2 of the heavier group ovulated. Animal no. 99, 45 days of age and weighing 13 gm., had one ovum without granulosa cells when killed after a delay of 44 hours. Ovulation in these 2 mice may be attributed to their greater age.

Upon decreasing the dosage to 30 I.U., only two animals weighing more than 12 gm. were injected. These did not ovulate. Neither did 20 I.U. cause follicles to rupture. The 3 animals ovulating after receiving 15 I.U. weighed 12 to 13.6 gm.

Injections of 10 I.U. produced no results in animals weighing less than 12 gm. In the heavier weight group, 5 of 12 animals responded. Several animals were examined at 50 hours but ovulation had not taken place. This again shows that a delay of longer than 24 hours before autopsy is unnecessary.

With 5 I.U., 13 of 15 animals weighing more than 12 gm. ovulated. This has been the most effective dose of chorionic gonadotropin injected thus far.

Ovulations were found in 4 of 5 animals weighing 14.7 to 17.5 gm. receiving 2.5 I.U. They were killed 21 to 22 hours after the injection.

In an effort to determine the minimal ovulating dose for chorionic gonadotropin a series of animals was injected with 2, 1.5, and 1 I.U. Only one each of the series receiving 2 and 1.5 I.U. ovulated. None of the other animals responded although they weighed between 12 and 15 gm. There was, however, definite follicular stimulation in most cases. These results indicate that the minimal ovulating dose of chorionic gonadotropin in the late immature mouse is 2.5 I.U. which is 2.5 times greater than that for the diestrous mouse (1 I.U.) and more than 3 times that for the pregnant mouse (0.7 I.U.). From a comparison of results shown in table 5, it would appear that 2.5 I.U. of the chorionic gonadotropin is as effective as 10 I.U. of pregnant mares' serum gonadotropin in inducing ovulation in late immature mice.

DISCUSSION

An examination of the literature shows so many factors to be involved in determining the effects of gonadotropic substances that it is small wonder conflicting reports have appeared. Attention of investigators has been called to such factors as methods of extraction, single or multiple substances in the gonadotropins, purity of the extract and equivalent amounts, dosage levels, frequency and routes of injections, lapse of time until examination, age, weight, species and strain of animals used, normal and hypophysectomized animals, the end-point of bioassay such as premature vaginal opening, ovarian and uterine weights, macroscopic and histologic aspects of the ovary and induced ovulation. Some of these methods of bioassay are of doubtful

TABLE 5. COMPARISON OF RESULTS WITH PREGNANT MARES' SERUM AND CHORIONIC GONADOTROPIN

	Dosage in International Units										
	100	50	25	20	15	10	5	2.5	2.0	1.5	1.0
Pregnant mares' serum											
Animals treated[1]	4(4)	4(3)	7(4)			9(7)	8(3)	6(2)	4(0)	3(0)	5(0)
No. of ova	5–9	3–8	1–11			1–32	7–17	6–18			
Chorionic gonadotropin											
Animals treated[1]	6 died	4(2)		2(0)	3(3)	12(5)	15(13)	5(4)	5(1)	4(1)	5(0)
No. of ova		1–5			1–6	1–4	1–10	5–10	1	2	

[1] Number of animals ovulating is indicated in parentheses.

validity, since Levin and Tyndale (1937) have shown that ovarian weight responses to follicle-stimulating substances involve a possible error of at least 100 per cent. Frank and Berman (1939) concluded that none of the gonadotropin bioassay methods (uterine and ovarian weights, ovarian histology, premature vaginal opening) is better than roughly quantitative. Whether or not the phenomenon of induced ovulation in the mouse can be of value in determining the potency of gonadotropins must remain for further investigations.

The experiments with adult diestrous mice show that the minimal ovulating dose of chorionic gonadotropin is approximately 1 I.U., and that 0.75 I.U. is subliminal.[2] Apparently 1 I.U. is as effective as 3 or even 100 I.U. in causing the ovulation of a normal complement of ova. Leonard (1933) found that the rabbit ovulated after 2 R.U. of chorionic gonadotropin which about equals the 1 R.U. per kg. of rabbit weight found by Friedman (1932) and Rowe, Simond and Nelson (1934). Hill and Parkes (1930) found no correlation between the dos-

[2] A recent investigation in this laboratory of the potency of another sample of chorionic gonadotropin (Follutein) shows there may be some variation in the ovulating capacity of different batches. The minimal ovulating dose for this latest sample was 2.25 I.U.

age and the number of follicles ovulating, and Jares (1932) could see no difference in the time of ovarian response following large or small doses. Similar conclusions may be made for the mouse.

The ova found in the ampullae approximately 18 hours after an injection of chorionic gonadotropin are not to be confused with those of a normal estrus. The small, thin uteri are typical of diestrus and the vaginal smears taken at the time of autopsy show that ovulation took place without the concomitant appearance of the cornified cells of estrus. Furthermore, the absence of spontaneous ovulations in those mice injected with 0.75 I.U. (table 1) supports the conclusion that the gonadotropin of larger doses is the primary cause of the ovulation.

Reports of induced ovulation in the pregnant rabbit have been fairly common but very few observations of this phenomenon have been recorded for the rat or mouse. Zondek and Aschheim (1928) found 3 pregnant mice had ovulated after receiving fresh anterior pituitary implants, but Engle and Mermod (1928) and Coca (1940) reported that ovulation in pregnant rats and mice occurred only after the termination of pregnancy. A report from this laboratory (Burdick and Whitney, 1941) showed that a single injection of untreated pregnancy urine induced ovulation in the pregnant mouse.

The results herein reported show that the pregnant mouse ovary is more sensitive to chorionic gonadotropin than that of the diestrous mouse. Three pregnant animals ovulated after single injections of 0.7 I.U. and 6 others after receiving 0.8 I.U. It is of interest to compare this slightly greater sensitivity of pregnant mice with reactions of other pregnant animals. Hill and Parkes (1932) concluded that it was not appreciably more difficult to induce ovulation in pregnant rabbits than in the non-pregnant animals. Rowlands (1935), however, found the minimal ovulating dose of either pregnancy urine or anterior pituitary extract to be 2 to 3 times greater for 15 to 25-day pregnant rabbits than for those in estrus. Snyder and Wislocki (1931) reported that the threshold for ovulation in the pregnant rabbit remains high compared to that of the non-gravid rabbit.

It appears difficult to attribute this ready response of mouse ovaries to anything but a direct action of chorionic gonadotropin on the ovaries in view of the fact that other investigators have found that the gonadotropic content of the anterior pituitary of various animals is reduced during pregnancy. Bacon (1930) reported that the gonadotropic content of the pituitary of cows was decidedly less in pregnancy, and J. Hill (1932) came to the same conclusion with parabiotic rats. Siegert (1933) also showed that the anterior pituitary of rats was less potent in pregnancy than in diestrus. Friedman (1929) and Wolfe (1931) found that the potency of the anterior pituitary of sows decreased during pregnancy, although Friedman observed that there was a marked variation. These variations, however, may paral-

lel or may even be induced by changes within the ovary (Fevold, 1939).

Several of our animals receiving adequate doses of chorionic gonadotropin failed to ovulate or yielded only 1 to 4 ova. This may be due to individual threshold differences, variations in absorption rates or possible refractory states of ovaries or pituitary. Dempsey (1937) has shown that although pregnancy does prevent preovulatory swelling of follicles and ovulation in the guinea pig, it does not interfere with the basic rate of growth of the follicles. He also concluded that FSH is secreted at a constant level but that LH is periodically released to cause ovulation. Swezy and Evans (1930) have established the fact that there is a succession of cycles of follicular growth throughout the gestation period in the rat. A number of follicles, varying from 2 to 12, increase in size in the usual 4 to 5-day periods, but they found no evidence of ovulation. It is possible that the injections in our animals which showed poor responses occurred at a time in the ovarian cycle when one group of follicles was degenerating and the new group had not yet sufficiently matured to rupture.

The important finding in this investigation is that a single subcutaneous injection of chorionic gonadotropin, regardless of its method of action, is responsible for the induction of ovulation in the mouse during pregnancy, that period in which ovulation does not normally occur. Of further importance is the fact that the pregnant mouse can also be of considerable value in the bioassay of ovulating substances in gonadotropic preparations.

Since it is generally considered that the effects of pregnant mares' serum gonadotropin differ from those of chorionic gonadtropin, it may be well to summarize the results with immature mice before taking up a further discussion. *a*) Single injections of extracts of both chorionic and pregnant mares' serum gonadotropin have the capacity to induce ovulations in immature mice 30 or more days of age. *b*) Weight of the experimental animal must be considered. *c*) Time is another important factor. Ovulation takes place within 24 hours after the injection. *d*) This response is generally dependent upon a proper dosage. *e*) The optimum response for pregnant mares' serum gonadotropin was found with 10 I.U. but for chorionic gonadotropin this was 5 I.U. *f*) No ovulations were found after doses of less than 2.5 I.U. of pregnant mares' serum gonadotropin nor after injections of less than 1.5 I.U. chorionic gonadotropin. *g*) Superovulations were seen after pregnant mares' serum gonadotropin but not with chorionic gonadotropin.

A comparison (table 5) of the capacities of pregnant mares' serum gonadotropin and chorionic gonadotropin to induce ovulations in mice weighing 12 gm. or more, shows that some ovulations occurred with pregnant mares' serum gonadotropin at all dosage levels from 100 I.U. to and including 2.5 I.U. Ovulation was not common with the

higher amounts of chorionic gonadotropin and doses as high as 100 and 200 I.U. caused death of the immature mice. The optimum response obtained with pregnant mares' serum gonadotropin was 10 I.U. (7 of 9 mice ovulated) and equally good results were obtained with only 2.5 I.U. of chorionic gonadotropin (4 of 5 mice ovulated). This indicates that an international unit of chorionic gonadotropin has an ovulational potency which is 2 to 4 times greater than that of pregnant mares' serum.

This investigation demonstrates the necessity of using several levels of doses to determine results. Ovulations would not have been obtained in immature mice had only the minimal ovulating dose for either diestrous or pregnant mice been used. Leathem (1939) found that ovarian responses of hypophysectomized immature rats to pregnant mares' serum were dependent upon dosage. Cole, Pencharz and Goss (1940) have stressed the necessity of using equivalent amounts of substances and a range of doses when comparing the action of gonadotropins. D'Amour and D'Amour (1940) found that larger doses of pregnant mares' serum caused a greater response (uterine weights and vaginal smears) than chorionic gonadotropin, but indicated that 1 I.U. of chorionic gonadotropin has approximately the same potency as 1 I.U. of pregnant mares' serum. After chemical and bioassays, Bischoff (1938) could detect no significant difference between the two.

Cole (loc. cit.) has reported superovulations in late immature rats several days after an injection of pregnant mares' serum. In the work reported here, superovulations were found with pregnant mares' serum within 24 hours, at 10, 5 and 2.5 I.U. levels but in no instance with chorionic gonadotropin at these levels. Carefully graded doses, however, must be tried before we can adequately compare the action of the two materials.

It is well known that the age of the experimental animal also affects the ovarian response. Saunders and Cole (1936) reported that induction of ovulation does not often take place until rats are 19 days of age. Selye, Collip and Thomson (1935) found there was little stimulation of rat follicles with either chorionic gonadotropin or anterior pituitary extracts before 18 days of age. By increasing the number of daily injections, M. Hill (1932) showed that 21-day-old mice ovulated after 4 daily injections of 0.1 cc. of chorionic gonadotropin and 14-day-old mice responded after 6 such injections. Although Delfs (1941) concluded that the response (uterine and ovarian weight) of immature rats is more dependent upon age than weight, Barlow and Sprague (1941) emphasized the importance of uniform weight as well as uniform age of animals used in bioassays. The present investigation shows the importance of weight in securing uniform results. In table 3, it will be seen that of the animals injected with 2.5, 5 and 10 I.U. of pregnant mares' serum, 12 of 23 mice weighing more than 12 gm.

ovulated, while only 2 of 12 less than 12 gm. responded. In table 4, 5 of 12 animals in the heavier group ovulated after receiving 10 I.U. of chorionic gonadotropin but none of the 5 weighing less than 12 gm. ovulated. In all of these series, most of the mice were 30 to 31 days of age. Probably the sensitivity of the ovaries to ovulation-inducing substances is governed by the same factor (factors) which controls the animal's growth.

Another factor which we wish to stress is the time interval, 18 to 24 hours, between the injection and the determination of results. It is possible that in the experiments of other investigators, involving multiple injections of gonadotropins, ovulations may have taken place long before the final results were determined at 72 to 120 hours. The subsequent history of ovarian changes may well depend upon how the ovary responded during the first 24 to 48 hours. An ovary in which the follicles have ruptured and corpora lutea of ovulation have developed will present a very different picture several days later than one in which there was initially only follicular stimulation followed by luteinization.

After obtaining augmentation effects and luteinization with a highly purified FSH fraction, Bischoff (1940) concluded that until a follicle-stimulating hormone is prepared which does not produce these multiple effects, it is unwarranted to ascribe these properties to another hormone. Having seen multiple effects with an extract of chorionic gonadotropin which are similar to the effects of pregnant mares' serum when equivalent doses are used, we are inclined to view these substances as being fundamentally alike in physiological reactions.

SUMMARY

Ovulation was induced in adult diestrous, pregnant, and late immature mice within 24 hours after a single subcutaneous injection of a chorionic gonadotropin. The pregnant mouse was found to be more sensitive to pregnancy urine extract than the diestrous animal in that the minimal dose was only 0.7 I.U. in the former, but 1 I.U. in the latter. There appeared to be no difference in reaction time or number of ova found following large or small optimal doses.

Dosage, and weight of the animal, were found to be important factors in the response of immature mice to pregnancy urine extract and pregnant mares' serum. Within the range of doses used, optimal responses were obtained with 10 I.U. of pregnant mares' serum, but with 5 and 2.5 I.U. of chorionic gonadotropin. No ovulations were seen after injections of less than 2.5 I.U. of pregnant mares' serum or of 1.5 I.U. of chorionic gonadotropin. These results indicate that, for these particular samples and according to their indicated ratings in international units, chorionic gonadotropin had 2 to 4 times the ovulating potency of pregnant mares' serum. When physiologically

equivalent doses were used, the ovarian responses to chorionic gonadotropin and pregnant mares' serum were similar except that the latter caused superovulation in a few animals. Weight of the immature mouse was found to be even more important than age. Very few of the animals 30 to 34 days of age weighing less than 12 gm. responded to treatment.

After ovulation in the mouse the ampulla of the oviduct is distended with the egg mass and fluid. Eggs may be easily counted. Induced ovulation in the mouse is such a definite end-point for a bioassay that it warrants further investigation as a rapid test of the potency of gonadotropins. It appears to be of definite value as an 18 to 24-hour pregnancy test.

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INHIBITION OF ESTROGENIC EFFECTS ON THE REPRODUCTIVE SYSTEM OF THE MALE RAT BY TESTOSTERONE INJECTIONS

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IT HAS recently been reported (Weichert and Kerrigan, 1942) that injection of estrogen into lactating female rats has a profound effect upon the reproductive systems of the suckling young. Daily injections of .1 mg. of diethylstilbestrol begun on the day of parturition bring about, among other things, a striking cornification of the epithelium of the prostatic utricle, prostatic urethra and ejaculatory ducts. The lining of the bulbus urethra and duct of Cowper's gland becomes a highly stratified squamous epithelium. These reactions are very evident by the 14th day of life. Freud (1933), Lacassagne and Villela (1933), Burrows and Kenneway (1934), Burrows (1935a, b), deJongh (1935), Korenchevsky and Dennison (1935), Overholser and Nelson (1935), Weller, Overholser and Nelson (1936), Wells (1936), Burrows (1937), Rusch (1937) and Harsh, Overholser and Wells (1939) have injected estrogens directly into older normal and castrated male rats, mice and ground-squirrels and have observed metaplastic changes in the epithelium of various parts of the reproductive system. The high dosage and long period of time over which injections were administered undoubtedly account for the findings in older animals. There is some indication in mice and rats that injections of testosterone suppress these pathological changes to some extent (deJongh, 1935; Korenchevsky and Dennison, 1935; Overholser and Nelson, 1935; Rusch, 1937; Harsh, Overholser and Wells, 1939).

In some preliminary experiments in which daily doses of .1 mg. of diethylstilbestrol were injected over a period of 10 days into normal and castrated adult male rats, no metaplastic changes in the accessory reproductive organs were noted. Two normal male rats 32 days old were given 10 daily injections of .05 mg. of diethylstilbestrol and autopsied on day 42. They showed no response to the injections save for a slight metaplasia of the epithelium of the duct of Cowper's gland. It seemed worthwhile to investigate the problem further in order to determine whether the accessory sex organs gradually lose their capacity to respond to diethylstilbestrol in the manner indicated above.

The present report is concerned with the effects of diethylstil-

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bestrol on the reproductive systems of immature normal and castrated male rats of various ages and with the inhibition or suppression of these changes by testosterone.

MATERIALS AND METHODS

The litters of 14 rats were reduced to 6 immediately after birth. Each litter of 6 contained at least 4 males. All of the males in 10 litters were castrated on the 5th day of life. Six males in one litter served as normal controls and two young were killed on each of days 21, 28 and 35. Six males of another litter castrated on the 5th day of life served as castrated controls and two young were killed on each of days 21, 28 and 35.

Only two males in each of the remaining litters were used for each type of injection. These consisted of *a*) normal rats injected daily for 14 days with .01 mg. of diethylstilbestrol; *b*) castrated animals injected daily for 14 days with .01 mg. of diethylstilbestrol; *c*) castrated animals injected daily for 14 days with .25 mg. of testosterone propionate and *d*) castrated animals injected daily for 14 days with both .01 mg. of diethylstilbestrol and .25 mg. of testosterone propionate. In each group two animals were injected from days 7 to 20 and autopsied on day 21; two animals were injected from days 14 to 27 and killed on day 28; two animals were injected from days 21 to 34 and autopsied on day 35.

At autopsy the reproductive tracts were removed *in toto* and fixed in Bouin's solution preparatory to histological study. Serial sections through the urogenital sinus region as well as of Cowper's glands and ducts and the adjacent part of the urethra were prepared.

OBSERVATIONS

In the normal male rat the proximal portions of the ducts of the seminal vesicles join the vasa deferentia to form the ejaculatory ducts which open into the prostatic portion of the urethra on the sides of the Müllerian tubercle. Müller's tubercle projects from the dorsal wall of the urethra. Between the openings of the ejaculatory ducts in Müller's tubercle lies the prostatic utricle (vagina masculina, uterus masculinus) which represents the remnant of the fused lower ends of the degenerated Müllerian ducts. The contiguous portion of the prostatic urethra receives many small ducts of the prostate gland.

The paired, compound-tubular Cowper's glands are small ovoid bodies, the ducts of which course for some distance before entering the bulbus urethra near the mid-line. An inferior bulbo-urethral gland is located near the point at which the ducts of Cowper's glands enter the urethra. In the rat, the inferior bulbo-urethral gland is a large, unpaired, median mass of glandular tissue located between the base of the penis and the bulbo-cavernosus muscle. It opens by many ducts into the bulbus urethra. Mossman, Lawlah and Bradley (1932) consider this structure to be an expansion of the ducts of Cowper's glands.

Comparison of these regions in normal males rats of 21, 28 and 35 days of age, shows that a gradual growth and differentiation occurs

in the various structures mentioned above but there is no striking change not associated with the normal development of the entire region. Examination of the same parts in castrated males 21, 28 and 35 days of age shows that full development of Müller's tubercle has occurred. Although the seminal vesicles and Cowper's glands have grown and show some degree of development, nevertheless they are much smaller than those of normal animals and their epithelia have not differentiated. The inferior bulbo-urethral gland is not significantly affected by castration, at least during the first 5 weeks of life. The anterior prostate glands of the castrated rats, although smaller than those of normal animals, have grown and differentiated (Price, 1936). The light secretory areas, located between the nucleus and lumen in the epithelium, are present but are not so consistent in occurrence nor so conspicuous as in normal animals of the same age.

Normal rats injected with diethylstilbestrol. The response of normal immature male rats injected daily for 14 consecutive days with .01 mg. of diethylstilbestrol varied with the age of the animal at the time when injections were begun. Thus, in animals injected from days 7 to 20 and killed on day 21, the epithelium of the duct of Cowper's gland showed a high degree of metaplastic stratification of squamous cells particularly in the portion where the duct leaves the gland. The epithelium of the bulbus urethra showed a slight degree of metaplastic squamous stratification in the immediate vicinity of the point at which Cowper's ducts enter. Cowper's gland itself was markedly inhibited both as to growth and differentiation. The inferior bulbo-urethral gland showed no change over the normal. The epithelium of the prostatic utricle was cornified but that covering Müller's tubercle and lining the prostatic urethra appeared to be unchanged. Although the ejaculatory ducts appeared normal, the proximal portion of the duct of the seminal vesicle showed a highly stratified squamous epithelium ranging from 7 to 14 cell layers in thickness. Actual cornification of this metaplastic epithelium had not taken place. The seminal vesicle proper and the anterior prostate gland showed a decided degree of inhibition but this was more obvious in the former than in the latter.

Normal rats injected similarly from the 14th to the 27th day of life and killed on day 28, showed less marked effects than did the younger animals described above. Growth of Cowper's gland was inhibited but its duct showed some squamous stratification. The inferior bulbo-urethral gland appeared somewhat smaller than normal. The epithelia of the prostatic utricle and duct of the seminal vesicle were slightly stimulated and the development of the seminal vesicle itself was inhibited. The anterior prostate had differentiated to a considerable degree but had not reached the state of development of the normal animal even though the characteristic light areas were present in the epithelial cells.

When injections of .01 mg. of diethylstilbestrol were given from

days 21 to 34 and the animals killed on day 35, the effects were still less obvious. The epithelium of the duct of Cowper's gland was but very slightly stimulated and the gland itself had developed but little. The inferior bulbo-urethral gland showed some inhibition in development. The epithelium of the prostatic utricle was very slightly stimulated but that of the duct of the seminal vesicle had not responded to the injections. The development of the seminal vesicle proper had been inhibited. The anterior prostate showed the typical light areas in the mucosal epithelium but it was not so large as in the normal animal.

Castrated rats injected with diethylstilbestrol. Castrated males injected with diethylstilbestrol in the same manner and for the same length of time as the normal animals described above, showed a much more striking response to the hormone than did those in which the testes were intact. In the 21-day-old animal injected for the preceding 14 days, Cowper's gland was like that of a typical castrated animal but its duct showed a highly stratified squamous epithelium. More striking, however, was the high degree of squamous metaplasia of the epithelium lining the bulbus urethra. The stratification even extended a short distance up some of the ducts of the inferior bulbo-urethral gland. Not only was the epithelium of the proximal part of the duct of the seminal vesicle stimulated but the ejaculatory duct showed a response as well. The epithelium of the prostatic utricle also was stimulated to a greater degree than is seen in the normal injected animal. The seminal vesicle proper showed no development beyond duct formation but the marked growth of the smooth muscle layer, first reported by Freud (1933) and Overholser and Nelson (1935), was striking. The anterior prostate gland was similar to that of the uninjected castrated control.

The castrated rats injected from days 14 to 27 and killed on day 28 showed the same responses as those just described. The results were in marked contrast to those obtained from similarly treated animals with intact testes.

In the older animals killed on day 35 the prostatic utricle, the ducts of Cowper's glands and the small ducts of the inferior bulbo-urethral gland showed similar responses to those described above but the effect on the epithelium of the duct of the seminal vesicle was decidedly less.

Castrated rats injected with testosterone and those injected with both diethylstilbestrol and testosterone. The castrated males injected daily for 14 days with both .25 mg. of testosterone propionate and .01 mg. of diethylstilbestrol were compared with those injected for 14 days with .25 mg. of testosterone propionate alone. All of these animals showed highly developed accessory reproductive organs. No effects from the diethylstilbestrol injections were observed and those animals injected with both hormones showed as great a degree of development of the accessory reproductive organs as those injected with testos-

terone alone. The effects on prostate and seminal vesicle are in accord with the findings of Thomson and Greene (1941) and Greene and Thomson (1942).

DISCUSSION

It appears from the results obtained here that normal immature male rats gradually lose the capacity to respond to injections of diethylstilbestrol, at least in such amounts as were utilized in these experiments. When injections are begun late in infancy the degree of stimulation of the epithelia of the prostatic utricle, ducts of the seminal vesicles and ducts of Cowper's glands is less than if injections are begun early in infancy. The inhibitory effects of diethylstilbestrol on the development of the epithelial of seminal vesicle, prostate and Cowper's glands also becomes less pronounced as the animals grow older. However, the capacity of the former group of structures to react to injections of the magnitude administered here, is not lost. This is shown by the positive responses of castrated animals treated in an exactly comparable manner. Only in the oldest group of castrated males was there a diminution in response. This was shown only by the decreased effect on the epithelium of the duct of the seminal vesicle and may be but a reflection of the increased size of the animal in relation to the amount of hormone injected. When testosterone in daily doses of .25 mg. is injected concomitantly with .01 mg. of diethylstilbestrol all of the effects of the latter are nullified. It may, therefore, be assumed that as the normal animal grows older it loses its capacity to respond to diethylstilbestrol because of the gradual increase in secretion of the male hormone by the intact testes.

These findings direct attention to the interesting question of the antagonistic effects of androgens and estrogens. Studies of others on the comb of the fowl (Morató-Manaro and Albrieux, 1939; Mühlbock, 1940), bones of mice (Gardner and Pfeiffer, 1938), skin of rats (Hooker and Pfeiffer, 1943) and reproductive systems of rats, mice and ground-squirrels (deJongh, 1935; Korenchevsky and Dennison, 1935; Overholser and Nelson, 1935; Rusch, 1937; Harsh, Overholser and Wells, 1939; Emmens and Parkes, 1938) indicate that androgen may inhibit the action of estrogen. On the other hand, investigations in which estrogens have been injected into normal adult rats with intact testes, have shown that metaplastic changes can be induced in the ducts of Cowper's glands, seminal vesicles, coagulating glands and even in the epithelium of the anterior prostate (Freud, 1933; Lacassagne and Villela, 1933; Burrows and Kenneway, 1934; Burrows, 1935a, b; deJongh, 1935; Korenchevsky and Dennison, 1935; Overholser and Nelson, 1935; Weller, Overholser and Nelson, 1936; Wells, 1936; Burrows, 1937; Rusch, 1937; Harsh, Overholser and Wells, 1939). Since in the latter experiments, the dose of estrogen was high and treatment was continued for long periods, it is obvious that antagonism in the ordinary sense did not occur. It seems to the authors that the response obtained from injecting estrogens and androgens

simultaneously depends more upon the relative concentrations of these hormones than upon such a phenomenon as antagonism. Thus the amount of each hormone injected and the length of time during which the hormones are administered will determine the result that will be obtained.

The correlation between the response of certain portions of the reproductive system of the male and their embryological derivation from Wolffian and Müllerian ducts is of interest. Lacassagne (1933) suggested on the basis of the response of the mouse prostate to estrogens that this structure might be a derivative of the Müllerian duct. Burrows (1935b) emphasized the possibility that the Müllerian ducts may contribute to the formation of the portion of the urogenital sinus into which they open since the prostatic ducts arising from this portion of the sinus react to estrogenic stimulation. Zuckerman (1940) in a comprehensive discussion of this subject, suggests that the caudal ends of both Müllerian and Wolffian ducts are invaded during early development by epithelium from the urogenital sinus which in itself is sensitive to estrogenic stimulation. Hence the similarity in response by squamous metaplasia of the prostatic utricle epithelium (ordinarily conceived to be a Müllerian duct derivative) and the epithelium of the ejaculatory duct or caudal portion of the duct of the seminal vesicle (considered to be derived from the Wolffian duct). The fact that in the female the epithelium of the lower portion of the Müllerian duct responds to estrogenic stimulation by squamous metaplasia, whereas the upper portion reacts in a different manner by glandular (secretory cell) proliferation, supports this contention. Burns (1942) working with the opossum, has reached similar conclusions.

On the other hand, the explanation for the squamous metaplasia of the epithelium of the duct of Cowper's gland in response to estrogen is more obscure. The opening of the duct of Cowper's gland into the bulbus urethra at some distance from the prostatic portion where the Müllerian and Wolffian ducts enter, makes it difficult, according to Zuckerman's conception, to associate the changes in the duct of Cowper's gland with those of the above mentioned structures. Zuckerman (1940) states that Cowper's glands, bulbar glands and penile portion of the urethra, generally considered to be endodermal in origin, are actually almost wholly of ectodermal derivation and therefore may respond in the same manner as the circumgenital skin. However, in the experiments reported here, the only part of the urethra proper which responded to estrogen was a localized area in the bulbous portion. It would seem that if Zuckerman's interpretation is correct more of the urethral epithelium should react to the diethylstilbestrol injections.

SUMMARY

Fourteen daily injections of .01 mg. of diethylstilbestrol were administered to immature normal male rats beginning on the 7th,

14th and 21st days of life. The animals were killed on the day after the last injection. Serial sections of the accessory sex organs were prepared for histological study. The squamous metaplasia observed in the epithelium of the ducts of Cowper's glands, prostatic utricle and caudal portions of the ducts of the seminal vesicles was found to vary with the age of the animal at the time when injections were begun. The older the animal at the time that injections were begun the less was the degree of stimulation of these structures. When the same procedure was followed in rats castrated on the 5th day of life a much more striking response to injections of diethylstilbestrol was obtained. Rats castrated on the 5th day of life and injected with both .01 mg. of diethylstilbestrol and .25 mg. of testosterone propionate showed that the testosterone completely prevented the diethylstilbestrol from exerting its action. It is suggested that as the normal animal gets older it loses its capacity to respond to estrogenic stimulation because of the gradual increase in secretion of androgen by the intact testes.

The bearing of these findings upon the questions of androgen-estrogen antagonism and the embryological derivation of certain estrogen sensitive tissues is discussed.

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BENEFICIAL EFFECT OF ESTROGENS ON ALTITUDE TOLERANCE OF RATS

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IT IS well known that exposure to atmospheric conditions producing anoxia causes enlargement of the adrenal cortex. That this process serves a useful compensatory function is indicated by observations recently made in this laboratory by Lewis, Davis, Thorn and Jones (unpublished data). In their experiments two doses of adrenal cortical extract were given to normal rats 12 hours and 2 hours, respectively, before exposure. These consisted of 0.5 cc. of a concentrate in sesame oil, representing 10 cc. of Kendall's aqueous adrenal cortical extract per cc., or 750 gm. of adrenal cortical tissue. As judged by survivals in single exposures this treatment produced an increase in the tolerance of rats towards atmospheric pressure so lowered as to cause anoxia. The beneficial effect was equivalent to an increase of altitude tolerance of about 2000 to 3000 feet. Since desoxycorticosterone did not exert this effect in identical experiments, it was considered probable that the benefit depends on the carbohydrate regulating factor of the adrenal cortex.

It has been found by several investigators that large doses of natural estrogens or the synthetic estrogen, diethylstilbestrol, produce in rats enlargement of the pituitary gland and adrenal cortex, and an increase in blood sugar and liver glycogen characteristic of increased adrenal cortical activity. Long (1942) and his collaborators (Fry, *et al.*, 1942) have shown in acute experiments, and Janes and Nelson (1942) in chronic experiments, that this effect on carbohydrate metabolism depends on the presence of the pituitary and the adrenals.

On the basis of this stimulating effect of estrogens on the adrenals it seemed worthwhile to test their effect on altitude tolerance, particularly since neither the adrenal carbohydrate-regulating factors nor pituitary adrenocorticotropin are available except in very limited quantities, whereas synthetic diethylstilbestrol is readily available and is relatively inexpensive. After it was found that estrogens produce a definite increase in altitude tolerance, two other steroid hormones, progesterone and testosterone, were investigated, but found to be without benefit. Finally the effect of diethylstilbestrol on the

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altitude tolerance of adrenalectomized rats was studied, with the unexpected findings of similar marked benefit.

PROCEDURE

Young adult male albino rats of the Wistar strain were used. A few experiments with male albino rats of the Sprague-Dawley strain and two experiments on groups of female rats were also made. In each experiment 10 control animals, injected subcutaneously daily with the appropriate oil (sweet almond oil in the controls for the stilbestrol experiments, sesame oil in the controls for the others) were subjected to reduced atmospheric pressure simultaneously with one or two groups of 10 treated animals which had been injected subcutaneously daily with the hormone. Fed, rather than fasted, animals were used because the former were considered in a state more nearly comparable to that of pilots or others exposed to conditions producing anoxia in aviation. In a few experiments no consistent difference was found between the mortality of 24-hour fasted and fed rats. Consequently, in the experiments here reported the animals were fed *ad libitum* up to the time of exposure to low pressure, and were not fed during the exposure.

A small steel decompression chamber containing an electric light and a glass observation window was used. The temperature of exposure varied between 24 and 29°C.; the humidity was also uncontrolled. The pressure in the chamber was gradually reduced over a period of 10 minutes, which is not included in the recorded duration of exposure. Adequate ventilation was maintained; at the lowest rate of ventilation used, the CO_2 content of the exhaust gas did not exceed 0.3 per cent.

The use of a standard period of exposure of fixed duration and pressure in preliminary experiments was found to produce such a wide variation in mortality that some experiments resulted in total mortalities approaching 0 per cent or 100 per cent, making a comparison of control and treated groups impossible. The duration and degree of exposure was therefore adjusted during each experiment in an attempt to secure a 50 per cent overall mortality, at which the sensitivity of the test would be expected to be maximal. The animals were exposed in all cases to a normal atmosphere and the pressure levels employed for exposure are referred to in terms of equivalent altitudes expressed in feet using the data of altitude-pressure tables based on the United States standard atmosphere (Brombacher, 1942) without correction for temperature.

Although this variation of the exposure ostensibly complicates the interpretation of the results, it seemed justifiable since each experiment had its own internal control. The rationale of this procedure depends on the assumption that the same factors are responsible for death within the range of duration and altitude of exposure here used: 1 to 3:40 hours and simulated altitude equivalent to 27,000 to 31,000 feet.

Glycogen in certain experiments was determined by the method of Good, Kramer and Somogyi (1933) modified in that the glucose produced was determined colorimetrically by the Folin method.

RESULTS

The data on the synthetic estrogen, diethylstilbestrol, are presented in table 1. Since this investigation was started on the assump-

TABLE 1. EFFECT OF DIETHYLSTILBESTROL ON MORTALITY OF RATS AT LOW PRESSURES

Expt.	Dosage, mg.	Duration Treatment, days	Av. Initial Weight, gm.	Av. Final Weight, gm.	Mortality Per cent	Exposure
1	Control	8	172 (150–198)	182 (160–208)	80	3¾ hr. 31,000 ft.
	0.05		171 (146–200)	168 (148–194)	40	
	0.05		165 (154–186)	161 (146–194)	40	
2	Control		183 (148–232)	185 (150–236)	90	3¾ hr. 31–29,000 ft.
	0.005	3	195 (150–232)	184 (138–222)	80	
	0.05		198 (158–226)	192 (156–222)	55 (9)	
3	Control	7	168 (142–216)	185 (146–238)	70	3¾ hr. 31–27,000 ft.
	0.002		158 (130–184)	156 (126–176)	33 (9)	
	0.02		170 (142–190)	163 (148–178)	50 (8)	
4	Control	7	134 (116–154)	147 (132–170)	70	2 hr. 30,000 ft.
	0.002		132 (118–148)	135 (118–158)	33 (9)	
5	Control	6	110 (100–118)	137 (124–146)	90	1½ hr. 30,000 ft.
	0.02		110 (102–120)	115 (105–131)	0	
6	Control	7	109 (100–118)	144 (136–152)	90	2 hr. 30,000 ft.
	0.02		113 (108–118)	115 (101–130)	0	
7	Control	6	126 (114–140)	139 (128–152)	70	3½ hr. 28–32,000 ft.
	0.02		126 (116–134)	124 (116–130)	50	
8	Control	6	122 (110–136)	134 (118–144)	40	2 hr. 30,000 ft.
	0.1		126 (114–134)	122 (108–128)	20	
Mean mortality of experiments on males (Expt. 1–6): Control 82%; treated 37%						
			Effect of doses below 0.001 mg.			
9	Control	7	164 (140–208)	176 (150–226)	55 (9)	2½ hr. 29–30,000 ft.
	0.0002		156 (134–182)	163 (144–190)	50	
	0.0006		146 (130–174)	149 (132–160)	60	
10	Control	7	134 (116–154)	147 (132–170)	70	2 hr. 30,000 ft.
	0.0006		120 (102–138)	131 (110–150)	30	
11	Control	5	162 (144–212)	165 (140–214)	20	2 hr. 28–30,000 ft.
	0.0002		161 (126–192)	165 (128–200)	50	
	0.0006		159 (128–192)	159 (132–190)	30	
			Effect of 24-hour treatment			
12	Control	1	250–300		30	2 hr. 27–29,000 ft.
	0.02				50	
13	Control	1	250–300		40	3 hr. 29–31,000 ft.
	0.5				50	

Each group represents 10 rats, minus an occasional obviously infected animal. The figure in parentheses following percentage mortality indicates the total number of animals in each experiment where more or less than 10 animals were used. The control of experiment 4 was also that of experiment 10. Experiments 7 and 8 were on female rats. Experiments 5–8 were on Sprague-Dawley rats, the rest were on Wistar rats.

tion that the carbohydrate-regulating factor of the adrenal cortex might be responsible for any beneficial effect, the first experiments were made with the large doses of diethylstilbestrol (0.5–0.05 mg. daily) reported by Ingle (1941a) to have a diabetogenic effect. These were injected daily for 3 to 7 days and proved to have a beneficial effect on altitude tolerance. No benefit was observed in experiments

TABLE 2. EFFECT OF NATURAL SEX HORMONES ON MORTALITY OF RATS AT LOW PRESSURES

Expt.	Dosage, mg.	Duration Treatment, days	Av. Initial Weight, gm.	Av. Final Weight, gm.	Mortality Per Cent	Exposure
			Estradiol Benzoate			
1	Control	7	118 (102–130)	130 (110–142)	90	2 hr.
	0.01		117 (96–128)	116 (96–138)	40	30,000 ft.
	0.05		111 (96–126)	106 (92–118)	20	
2	Control	7	139 (130–158)	144 (132–168)	67 (9)	2 hr.
	0.01		143 (124–190)	143 (124–178)	50	30,000 ft.
	0.05		136 (128–150)	130 (118–144)	33 (9)	
3	Control	7	144 (128–164)	155 (138–194)	60	1½ hr.
	0.01		144 (130–152)	142 (128–152)	30	29,000 ft.
	0.05		153 (130–190)	140 (114–176)	20	
Mean mortality: Control 72%; treated 32%						
			Testosterone propionate			
1	Control	7	154 (134–172)	159 (142–180)	50	2 hr.
	0.02		154 (126–174)	157 (134–172)	80	30,000 ft.
	0.2		152 (120–186)	161 (136–190)	80	
2	Control	7	135 (114–146)	145 (128–154)	80	2 hr.
	0.02		133 (120–156)	141 (126–168)	70	29–30,000 ft.
	0.5		131 (120–150)	145 (128–160)	50	
3	Control	7	150 (128–170)	150 (128–168)	20	2 hr.
	0.5		143 (130–158)	147 (130–166)	80	28,000 ft.
Mean mortality: Control 50%; treated 72%						
			Progesterone			
1	Control	7	140 (126–162)	144 (118–178)	33 (9)	2 hr.
	0.02		135 (120–154)	141 (128–162)	80	30,000 ft.
	0.2		141 (122–156)	149 (126–170)	100	
2	Control	7	145 (120–178)	160 (138–188)	90	2 hr.
	0.02		141 (128–156)	155 (130–184)	90	29–30,000 ft.
	0.2		151 (132–160)	158 (146–174)	70	
3	Control	7	150 (128–170)	150 (128–168)	20	2 hr.
	0.2		149 (136–168)	147 (138–166)	40	28,000 ft.
Mean mortality: Control 48%; treated 76%						

The control for testosterone, expt. 3 is also that for progesterone, expt. 3. The figure in parentheses following percentage mortality indicates the total number of animals used in each experiment where more or less than 10 animals were used.

made one day after a single injection (experiments 12 and 13). Because of the toxic effects (weight loss, rough fur, apathy), progressively lower doses were tried, with diminution, but not disappearance, of the toxic symptoms in doses as low as 0.002 mg. Lowered mortality was observed in all 9 of the groups of males treated with doses between 0.5 and 0.002 mg. of diethylstilbestrol. Increased tolerance was also observed, but was apparently less pronounced, in the two groups of females (experiments 7 and 8), but the data are insufficient to justify comparison between the sexes. In 5 groups treated with

0.0002 or 0.0006 mg. (approximately the estrogenic dose) weight gain was about equal to that in the controls, but there was no consistent increase in altitude tolerance. It was concluded that this drug was beneficial to altitude tolerance only in dosages which interfered with the normal rate of growth.

Organ weights observed in some of the experiments corroborated the adrenal hypertrophy, thymic atrophy and testicular atrophy (unrecorded) which have been reported by many workers following administration of estrogenic hormones.

Data relating to the experiments using estradiol benzoate are

TABLE 3. EFFECT OF DIETHYLSTILBESTROL ON MORTALITY OF ADRENALECTOMIZED RATS AT LOW PRESSURE

Expt.	Dosage, mg.	Duration Treatment, days	Initial Weight, gm.	Final Weight, gm.	Mortality Per Cent	Exposure
1	Control	6	171 (152–206)	187 (172–220)	89 (9)	2 hr.
	0.05		191 (162–216)	195 (172–224)	72 (7)	25–30,000 ft.
2	Control	7	159 (136–228)	170 (142–242)	89 (9)	2 hr.
	0.02		158 (132–222)	148 (126–212)	25 (8)	28,000 ft.
3	Control	7	169 (152–186)	172 (156–188)	40	3 hr.
	0.02		162 (142–206)	147 (118–190)	0 (11)	27–28,000 ft.
4	Control	7	139 (114–164)	144 (122–176)	72 (11)	1 hr.
	0.02		138 (116–156)	128 (114–146)	0 (11)	27,000 ft.
5	Control	7	139 (110–168)	174 (144–204)	64 (11)	1½ hr.
	0.02		132 (106–168)	134 (108–162)	27 (11)	26–29,000 ft.

Mean mortality: Control 71%; treated 25%

Expt. 5 was on Sprague-Dawley rats, the rest on Wistar rats. The animals of experiment 1 were adrenalectomized 8–10 days before treatment was started, the rest 0–4 days. The figures in parentheses following percentage mortality indicate the total number of animals used in each experiment where more or less than 10 animals were used.

presented in table 2, in which it is seen that a similar benefit was observed in all 6 treated groups. Those animals receiving the higher dosage of 0.05 mg. lost weight and those receiving the lower dosage, 0.01 mg. barely maintained weight; it was our impression that these animals appeared somewhat healthier than those treated with similar quantities of stilbestrol. In one experiment using .05 mg. the expected thymic atrophy was noted, but without adrenal hypertrophy.

Also presented in table 2 are the data on the effect of *a*) 0.02 to 0.2 mg. of progesterone, after which mortality was reduced in only 1 of 5 treated groups, and *b*) 0.02 to 0.5 mg. of testosterone propionate, which increased the altitude tolerance in only 2 of 5 treated groups. The increase in mean mortality of the groups treated with either of these hormones suggests that they may even be hamrful to altitude tolerance. Both of these hormones in the dosage studied produced slight thymic atrophy and no adrenal hypertrophy.

To test whether the beneficial effect of estrogens on altitude tolerance is mediated by the adrenals, 10 groups of 10 rats each were bilaterally adrenalectomized and kept alive by the subcutaneous implantation of a pellet of crystalline desoxycorticosterone acetate, which apparently does not affect carbohydrate metabolism or altitude tolerance in the intact rat. The data are presented in table 2. The treated animals were given .05 or .02 mg. of diethylstilbestrol, a dose which produced beneficial effect in normal animals. The controls gained weight at a normal rate on the stock diet employed at the National Institute of Health which contained 0.5 per cent NaCl in addition to that present in the cereal and meat components. In all 5 experiments the treated animals unexpectedly showed a decrease in mortality as compared with adrenalectomized controls. The 'ceiling' for adrenalectomized rats appears to be 2000 to 3000 feet less than that of normal animals.

The conclusion that these experiments prove the beneficial effect of diethylstilbestrol to be independent of the adrenals may be subjected to the criticism that rats often have accessory adrenal cortical tissue which might regenerate more rapidly under the influence of estrogen treatment. Accordingly in experiments 2, 3 and 4 of table 3, the completeness of adrenalectomy was tested by removing the pellet of desoxycorticosterone acetate from the survivors. Of the 37 animals so studied, 78 per cent died within 1 to 3 weeks, the remainder surviving for at least 4 or more weeks. This indicates 22 per cent as the maximum which may have possessed accessory adrenal cortical tissue in significant amount. This figure is considered maximal because it is reasonable to suppose that the rats dying during periods of anoxia would have fewer adrenal rests than those surviving. The number of adrenalectomized animals surviving without pellets represented only 16 per cent of the total number of animals exposed. The difference in altitude survival of 46 per cent noted between the treated and untreated groups is significantly greater than the 16 or 22 per cent possibly possessing adrenal rests, and so it is concluded that the treatment was of benefit in the absence of the adrenals.

Carbohydrate studies were undertaken as an independent check on the adequacy of adrenalectomy. Four adrenalectomized and four normal rats were treated daily for a week with 0.02 mg. of diethylstilbestrol and similar control groups with oil. The animals were fasted for 24 hours after the last injection and then killed. It is seen from the data in table 4 that the adrenalectomized animals had equally low levels of blood sugar and liver glycogen whether treated or not, whereas the intact animals had higher levels which were further increased by diethylstilbestrol. It is concluded that diethylstilbestrol exerted its beneficial effect on anoxia tolerance in spite of the absence of adrenal regulation of carbohydrate metabolism.

DISCUSSION

These experiments appear to offer adequate evidence that in intact or adrenalectomized male rats estrogens increase the tolerance to diminished oxygen pressure. The test used is a crude one dependent on mortality of treated animals in comparison with simultaneously exposed and untreated groups of animals. Mortality in such experiments fluctuates widely. Without data on a large number of such groups to establish the standard deviation of the mortality of normal animals subjected to a fixed exposure, one cannot say how many such exposures are necessary to establish the significance of a given mortality difference between treated and control groups. Each exposed group of animals, however, may be treated as a unit with an

TABLE 4. CARBOHYDRATE LEVELS OF RATS FASTED 24 HOURS AFTER 7 DAYS OF INJECTION OF 0.02 MG. OF DIETHYLSTILBESTROL

Animal	Blood Sugar mg. %	Liver Glycogen %
	Control oil; unoperated	
1	102	0.071
2	107	0.129
3	106	0.085
4	71	0.034
Mean	97	0.080
	Hormone treated; unoperated	
1		0.233
2	125	0.066
3	133	0.063
4	140	0.190
Mean	133	0.138
	Control oil; adrenalectomized	
1	61	<0.01
2	50	<0.01
3	105	<0.01
4	131	<0.01
Mean	87	<0.01
	Hormone treated; adrenalectomized	
1	77	<0.01
2	87	<0.01
3	97	<0.01
4	87	<0.01
Mean	87	<0.01

Rats of Sprague-Dawley strain. The original Good-Kramer-Somogyi technic for determining liver glycogen was used, without immersion of the liver in liquid air, which may account for the rather low levels found. The values represented as <0.01 denote an indetectable concentration of glycogen.

internal control. It is then seen that an increase in altitude tolerance was shown in all 11 of the groups treated with diethylstilbestrol in adequate dosage and in all 6 of the groups treated with estradiol benzoate. Similar consistent benefit is shown by the 5 groups of adrenalectomized animals treated with diethylstilbestrol. In contrast, the data presented on testosterone and progesterone, although inadequate to prove definitely either benefit or harm, certainly fail to show the degree of benefit characteristic of estrogens.

Although this study was originally undertaken on the basis of the widely held theory that the adrenal cortex plays a predominant rôle in the metabolic adjustments to anoxia (Lewis, *et al.*, 1942), these data have led us to the conclusion that adrenal and carbohydrate metabolism represent only part of the metabolic factors concerned in resistance to diminished oxygen pressure. Our experiments furnish no basis for deciding whether the site of action of estrogens in relation to altitude tolerance is peripheral or is mediated by the pituitary, as seems possible. One common feature associated with the increased altitude tolerance produced by adrenal cortical hormone, estrogens, or previous exposure, is atrophy of the thymus. Although no thymus weights were recorded on our adrenalectomized rats, as these were used for survival studies, it has been reported (Ingle, 1941a) that estrone causes thymic atrophy in adrenalectomized as well as in normal rats, and it is probable that stilbestrol does likewise.

Ingle (1941b) has shown that the weight loss caused by diethylstilbestrol is due to loss of appetite, for it may be prevented by forced feeding. In these experiments the weight loss was as great in the adrenalectomized as in the intact treated rats. The loss of appetite is therefore apparently not caused either by increased carbohydrate levels brought about by adrenal activity or by stimulation of any other adrenal function.

It does not seem likely that the beneficial effect of estrogens on altitude tolerance of adrenalectomized animals maintained on desoxycorticosterone acetate is due to any ability of estrogens to replace the life-maintaining hormones of the adrenal cortex in view of the report (Schacher, *et al.*, 1937) that estrogens are toxic to adrenalectomized animals (without desoxycorticosterone acetate) and diminish their post-operative survival. Our experience has confirmed this in that the stilbestrol-treated animals tended to die more rapidly after removal of the pellet than did the controls.

Whether these facts offer any hope of eventual practical application to the problems of aviation medicine seems doubtful, since the beneficial effect of estrogens is produced only by toxic doses. Mere enhanced ability to survive exposure to lowered oxygen pressure is of no practical significance to aviators, for what is needed is greater functional efficiency in flight at high altitudes. The treated animals

were more lethargic, and it is indeed possible that their relative inactivity during exposure was a factor in their greater survival.

Since we have no idea of the mode of action of estrogens in relation to altitude tolerance, we do not know whether they are effective in spite of, or possibly even by virtue of, their toxic action. It is to be hoped that compounds may be found which will produce the increase in altitude tolerance without the toxic or sexual effects of the estrogens.

SUMMARY

Normal and adrenalectomized adult male rats were exposed to normal atmospheres at lowered barometric pressures in a decompression chamber. Exposure was so adjusted with respect to pressure and duration as to give an overall mortality as close to 50 per cent as possible. In each experiment the mortality rate of control and treated animals was compared directly.

Subcutaneously injected diethylstilbestrol and estradiol increased the survival rate of normal adult male rats exposed to simulated altitude under critical pressure-time conditions. This beneficial effect was observed only with dosages which interfered with normal weight gain. Progesterone and testosterone failed to produce the improved altitude tolerance, but did not interfere with normal weight gain.

Adrenalectomized rats injected subcutaneously with diethylstilbestrol showed an increased survival rate under exposure to simulated altitude, as compared with adrenalectomized controls. It is concluded that the beneficial effect of diethylstilbestrol is not mediated exclusively by the adrenal glands.

We are indebted to the Upjohn Company, Kalamazoo, Mich., for the supply of stilbestrol in sweet almond oil.

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EFFECT OF GONADOTROPIC HORMONES ON INTRAOCULAR PROSTATIC IMPLANTS IN THE MALE RABBIT[1]

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IN A previous preliminary communication by Rosenberg, Krichesky and Benjamin (1941) it was reported that administration of gonadotropic hormones to male rabbits produced a consistent response in intraocular prostatic implants which to the writers' knowledge had been unreported. Two separate periods of growth and regression of prostatic implants occurred, the first termed primary response and the second termed secondary response.

The data obtained from the continuation of these studies confirmed the earlier observations. Subsequently a wider range of dosages was used and these results are now reported.

METHOD

Following the technic described by Benjamin, *et al.* (1940), 29 young adult male rabbits (4 to 7 months of age) were completely prostatectomized and bits of the excised tissue implanted into the anterior chamber of the eyes. Two implants were placed into each eye and of the 116 implants thus made, 84 were successful. Approximately 3 weeks were allowed for biological organization of the transplants.

Before the injections of gonadotropins the average area of each transplant was determined by photographing it 3 times per week for at least 2 weeks with a specially constructed Contax camera with fixed focus under constant illumination. The square area was determined from a tracing of each photograph by a planimeter.[2] The average of these was taken as 100 per cent and was considered the normal area. A subsequent increase or decrease in area was then expressed as greater or less than 100 per cent. All data were plotted on coordinate paper.

Four groups of animals were treated as follows: A, 15 were injected with varying doses of chorionic gonadotropin;[3] B, 14 were injected with varying

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[1] Aided by research grants from the Del Amo Foundation and the Board of Research, University of California.

[2] This method proved to be accurate to ±3%.

[3] Chorionic gonadotropin (APL) and the anterior hypophyseal gonadotropic factor, prepared by Ayerst, McKenna and Harrison (U. S.), Ltd., Rouses Point, N. Y.

doses of hypophyseal gonadotropin;[3] C, after approximately one month's rest, 6 animals from group A and 6 animals from group B were given a second series of injections with the same hormone used in each of the initial series, and D, after approximately one month's rest 7 animals from Group A, and 6 animals from group B were given a second series of injections with a different gonadotropic hormone from that used in the initial series. Each series of injections consisted of subcutaneous administration of hormone 6

TABLE 1. RESPONSE OF INTRAOCULAR PROSTATIC IMPLANTS TO CHORIONIC[1] AND HYPOPHYSEAL[2] GONADOTROPIC HORMONES

First Series of Injections				Second Series of Injections		
Animal No.	Dose/kg.[3]	Prim. resp.	Second resp.	Dose/kg.[3]	Prim. resp.	Second resp.
A1	500[1]	++++	++++	300[2]	++	+++
A2	500[1]	++	++			
A3	500[1]	++	+++	300[2]	+++	0
A4	500[1]	+++	++++	300[1]	0	++++
A5	500[1]	+++	+++	300[2]	+++	++++
A6	300[1]	++++	++++	300[2]	++++	+++
A7	300[1]	+++	++++	300[1]	−−	+++
A8	300[1]	++++	+++	200[1]	−−−	+++
A9	200[1]	++++	+++	200[1]	0	++
A10	200[1]	+++	+++	200[2]	+++	+++
A11	100[1]	++	0	100[1]	−−	++
A12	100[1]	++	+++	100[1]	−	+++
A13	100[1]	+++	++	200[2]	+	++++
A14	30[1]	++	++	100[2]	+++	++
A15	30[1]	++	0			
G1	500[2]	++++	++++			
G2	500[2]	++	++++	300[1]	+++	+++
G3	500[2]	++++	++	300[1]	++	+++
G4	500[2]	+++	+++	300[2]	−−−	+++
G5	300[2]	++++	++++	300[2]	+	++
G6	300[2]	+++	0	300[1]	++	0
G7	300[2]	++	++	200[2]	−−	+++
G8	200[2]	++	+++	200[1]	++++	+++
G9	200[2]	++++	+++	200[2]	0	++++
G10	200[2]	+++	++	200[1]	++	++++
G11	100[2]	+++	++	200[1]	+	++
G12	100[2]	++	0	100[2]	0	++
G13	50[2]	++	+++	100[2]	−	++
G14	50[2]	+	++			

[1] Chorionic gonadotropin indicated by[1].
[2] Hypophyseal gonadotropin indicated by [2].
[3] All injections were given subcutaneously 6 times per week for two weeks. Magnitude of response is indicated by + for an increase in area and − for a decrease in area as follows: ± = 10 to 15%; ± ± = 16 to 25%; ± ± ± = 26 to 35%, ± ± ± ± = over 36%. Units of dosage in international units for chorionic gonadotropin and in rat units for hypophyseal gonadotropin.

times per week for a 2-week period in doses varying from 30 to 500 I.U. of chorionic gonadotropin and 50 to 500 R.U. of hypophyseal gonadotropin per kg. per injection.

All pertinent data concerning the treatment of each animal are included in table 1.

RESULTS

Group A. In every animal in this group the prostatic implant areas increased immediately after the beginning of injections cho-

rionic gonadotropin (table 1). This increase reached a peak at 4 to 8 days after the beginning of treatment and averaged 31 per cent, with variations from 12 to 51 per cent. The lower doses (30 and 100 I.U. per kg., per injection) did not produce as great a response as did higher doses. There was a decrease in area of the prostatic implants, following the initial increase, despite continued injections, to an area usually larger than, although approaching that of, the implant prior to injection (fig. 1, animal A6).

A second increase in transplant area occurred 6 to 18 days after

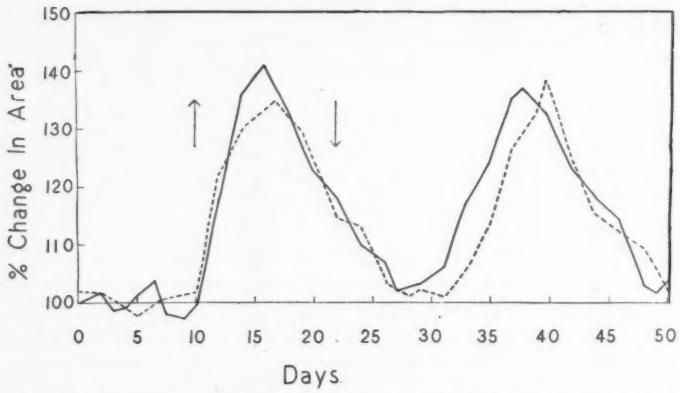

FIG. 1. Changes in area of intraocular prostatic implants on administration of a single series of 12 injections of gonadotropin. Animal A6, represented by the solid line, was given 300 I.U./kg. per injection of chorionic gonadotropin. Animal G5, represented by the broken line, was given 300 R.U./kg. per injection of hypophyseal gonadotropin. Arrow pointing up indicates the day injections were begun; arrow pointing down, the day injections were stopped.

the last injection in 13 of the 15 animals in this group (table 1). This increase approached and even exceeded, in some cases, the initial response (fig. 1, animal A6). The average magnitude of this secondary response was 28 per cent with a range of from 11 to 46 per cent. This was followed always by regression, approaching the normal area within 16 days after its peak.

Group B. In the 14 animals of the group injected with hypophyseal gonadotropin there was an initial or primary response of growth and regression in the intraocular prostatic implants not observably different either quantitatively or qualitatively from that in the animals in group A (table 1 and fig. 1, animal G5). A secondary response also occurred in all but 2 animals in this group as in the case of animals of group A.

Group C. Approximately one month after the first series of injections 6 animals from group A (A4, A7, A8, A9, A11, A12) were given a second series of 12 injections with chorionic gonadotropin in

the dosages indicated in table 1. Six animals from group B (G4, G5, G7, G9, G12, G13) were given a second series of 12 injections with hypophyseal gonadotropin (table 1). Only one (G5) of the above animals showed a slight prostatic growth response to this second series of injections and this reached a maximum of only 12 per cent; 4 (A4, A9, G9, G12) showed no response (fig. 2, animal G9) and 7 animals (A7, A8, A11, A12, G4, G7, G13) responded by a decrease in prostatic implant area ranging from 11 to 31 per cent (fig. 2, animal A7). Four to 13 days after the last injection, every animal exhibited an

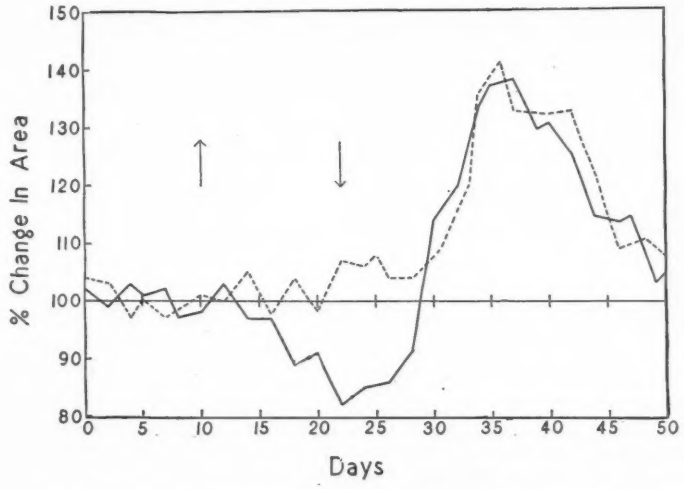

FIG. 2. Changes in area of intraocular prostatic implants on administration of a second series of 12 injections of gonadotropin into animals injected previously with the same gonadotropic agent. Animal A7, represented by the solid line, was given 300 I.U./kg. per injection of chorionic gonadotropin. Animal G9, represented by the broken line, was given 200 R.U./kg. per injection of hypophyseal gonadotropin. Arrow pointing up indicates the day injections were begun; arrow pointing down, the day injections were stopped.

increase in transplant area, the average being 24 per cent at the peak with variation from 17 to 39 per cent. This was followed by a regression to the pre-injection area within 25 days after the last injection. Thus, a second series of injections with the same gonadotropic agent elicited no primary response but a secondary response always was obtained.

Group D. Seven animals from group A (A1, A3, A5, A6, A10, A13, A14) and six animals from group B (G2, G3, G6, G8, G10, G11) were given a second series of 12 injections with a different gonadotropic agent from that used in the first series. The hormones used and the dosages employed are given in table 1. In every animal a primary response was obtained similar in duration and magnitude to the

initial response to a single series of injections. All of these animals except two (A3 and G6) also exhibited a secondary response no different from that obtained with the first series of injections (fig. 3, animals A5 and G8).

DISCUSSION

The initial or primary response to the series of gonadotropic hormone injections reported herein showed several consistent characteristics. There was a precipitous increase in prostatic implant area within 36 hours after the first injection. This increase reached a maxi-

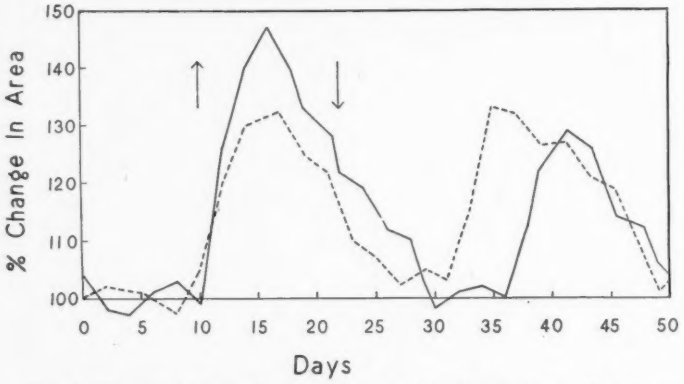

FIG. 3. Changes in area of intraocular prostatic implants on administration of a second series of 12 injections of gonadotropin into animals injected previously with a different gonadotropic agent. Animal G8, represented by the solid line, was given 200 I.U./kg. per injection of chorionic gonadotropin. Animal A5, represented by the broken line, was given 300 R.U./kg. per injection of hypophyseal gonadotropin. Arrow pointing up indicates the day injections were begun; arrow pointing down, the day injections were stopped.

mum 4 to 8 days after the beginning of treatment (fig. 1, 3). The decline in transplant area was more gradual and required from 10 to 16 days to approach the pre-injection area. In no instance did the maximum increase in area exceed 51 per cent above the normal area. A similar situation held for the secondary response (fig. 1, 3) in that the increase in transplant area was always more rapid than the regression and was of the same order of magnitude as the primary response.

The decline in area of intraocular prostatic implants, beginning immediately after the peak was attained, occurred regardless of continued hormone administration. It is probable that this regression may have been due to the appearance of antihormones despite the short periods of injection. The decline during the secondary response was similar in character to that during the primary but can hardly be explained on the basis of antihormones since no injections were made

in that period. It was suggested, therefore, that regression during the primary response may be due to different causes than the regression seen during the secondary response.

The magnitude and duration of the primary and secondary response were similar after the injection of either chorionic or anterior hypophyseal hormone. This was particularly true if the doses were above 30 I.U. per kg. of chorionic gonadotropin or 50 R.U. per kg. of hypophyseal gonadotropin.

It is interesting to note that the primary response obtained in this study is approximately equal in magnitude and duration to that obtained by Moore, *et al.* (1937). These workers administered 100 I.U. of gonadotropic substance from pregnancy urine daily for only 3 days yet the percentage increase in area, the time that the maximum area was reached, and the regression that followed the peak of growth are strikingly parallel to the results reported herein. They obtained, as we have confirmed, a similar primary response to injections of a hypophyseal gonadotropin. These data are not in agreement with those of Heckel and Kretschmer (1935) who reported continuous growth of intraocular prostatic implants for several months on administration of pregnancy urine gonadotropin.

Moore, *et al.*, (1937) reported that a second series of 3 injections of gonadotropins given up to 2 months after the first series elicited no primary response in intraocular prostatic implants. Our data confirmed this finding. These authors offered no suggestion as to the mechanism involved and considered the phenomenon only as confirmation of the general thesis of resistance to successive injections of this hormone. To determine whether this resistance was due to an antihormone, we employed two animals giving each of them 12 injections of 200 I.U. of gonadotropic hormone over a two-week period. They were then bled, and the serum collected. Ten cc. of this serum was injected into an untreated animal simultaneously with 100 I.U. of pregnancy urine hormone on 3 successive days. The administration of the serum did not prevent growth of prostatic implants. The short period of injection given by Moore, *et al.*, and the results of the one experiment cited here suggested but did not prove that either antihormones were absent or were not present in sufficiently high concentration to inhibit the action of gonadotropic hormone in the dosages used.

An adequate explanation for the primary and secondary growth responses reported here is not possible at this time. One hypothesis may be suggested which in the opinion of the writers possessed some merit. The administration of gonadotropic hormones stimulates male hormone production which results in growth of prostatic implants thus accounting for increased area during the primary response. Regression which follows may be due to the accepted phenomena involved in the resistance to successive injections of these hormones.

During the injection period it is possible that the endogenous gonadotropic hormone release was reduced or inhibited. When this effect was removed (several days after cessation of hormone treatment) the endogenous gonadotropins may again exert their effects causing the increase in prostatic implant areas seen in the secondary response. The regression occurring in the secondary response may be due to restoration of those factors that maintain equilibrium in the normal animal. Such an hypothesis offered a tentative explanation for the appearance of a primary and secondary response to a single series of gonadotropic injections. In addition it offered a possible explanation for the absence of a primary response to a second series of injections with the same hormone (resistance to successive injections) which did not interfere with the development of a secondary response. The use of a different gonadotropic agent in a second series of injections did exhibit a response similar to a single series of injections.

SUMMARY

Injections of either chorionic or hypophyseal gonadotropins caused a precipitous increase in intraocular prostatic implant area which reached a peak 4 to 8 days after the beginning of injections. Prostate areas then underwent a more gradual regression in spite of continued hormone administration. Six to 18 days after cessation of injection a second increase in the transplant area occurred which equalled or even exceeded the initial rise. The two periods of growth and regression are termed primary and secondary response, respectively. A second series of injections with the same gonadotropic agent did not produce a primary response but was followed by a secondary response. Administration of a second series of injections with a different gonadotropic agent than that used in the initial series produced both a primary and a secondary response not observably different from that which followed the first series of hormone injections. An hypothesis was suggested to explain these responses.

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ANDROGENS AND EXPERIMENTAL MENSTRUATION IN THE MONKEY (*Macaca mulatta*)

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SEVERAL of the physiological reactions produced by testosterone closely resemble those characteristic of progesterone. Among these are certain effects on the primate endometrium. Its administration will inhibit menstruation in normal monkeys (Hartman, 1937; Zuckerman, 1937) and women (Gaines, Salmon and Geist, 1938; Papanicolaou, Riley and Shorr, 1938), prevent estrogen-withdrawal bleeding in monkeys (Hartman, 1937; Engle and Smith, 1939; Duncan, Allen and Hamilton, 1941) and bleeding following castration (Hartman, 1937). The development induced by estrogen in the endometria of castrated monkeys is maintained by testosterone without involution or progestational changes, and when bleeding is inhibited following injections of progesterone, the progestational condition of the endometrium is preserved (Engle and Smith, 1939). In contrast with these reactions, testosterone has an estrogenic action on the sexual skin (Hartman, 1937; Engle and Smith, 1939; Duncan, Allen and Hamilton, 1941). It is also notable that for these effects the amount of testosterone required is in all instances comparatively large.

It has been found recently that bleeding can be precipitated in castrated monkeys receiving estrogen by injecting small amounts of progesterone. Animals receiving 1000 I.U. daily for a sufficient time usually bleed within 5 to 10 days after the injection of 1 mg. of progesterone (Hisaw, 1942). Such sensitivity to progesterone seemed to furnish an additional means for comparing the effects of progesterone and testosterone on the endometrium.

MATERIALS AND METHODS

Fifteen adolescent monkeys (*Macaca mulatta*) weighing from 3.0 to 3.5 kg. and 1 adult (114) weighing 4.7 kg. were used in these experiments. All were castrated with the exception of 6 of the smaller animals which were definitely sexually immature. Each animal was given a priming treatment with about 1000 I. U. of estradiol (8.33 μg.) subcutaneously in 0.25 cc. of sesame oil daily for 30 days or longer before receiving testosterone or testosterone propionate. Most of the animals were used repeatedly, and when bleeding occurred, the injections of estrogen were discontinued during the period of active menstruation.

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Seven of the smaller monkeys were used in a preliminary experiment to test the ability of testosterone propionate to inhibit bleeding following the withdrawal of estrogen at the end of the priming treatment. The point sought was not the amount that would delay bleeding but rather the minimal quantity required to inhibit estrogen-withdrawal menstruation for at least 20 days. Testosterone propionate was given subcutaneously in daily doses of 1.0 to 3.0 mg. (table 1). The 3 monkeys receiving 1 mg. daily bled between the 9th and 11th days; and of the 3 given 2 mg. daily, 1 bled on the 11th day and 1 on the 21st, while the third had not bled by the 25th day but did so 6 days after the injections were stopped. The animal receiving 3 mg. daily had not bled by the 20th day, at which time the dose was reduced to 1 mg. daily, and bleeding occurred during treatment 19 days later.

These few data indicate that a daily dose of 2.0 mg. of testosterone propionate is approximately the minimal amount required to inhibit bleeding

TABLE 1. INHIBITION OF ESTROGEN-WITHDRAWAL BLEEDING BY TESTOSTERONE PROPIONATE

Animal No.	Days of Pretreatment with 1000 I.U. of Estrogen	Daily Dose of Testosterone, Propionate mg.	Day Bleeding Began
100	32	1	9
103	28	1	9
105	58	1	11
106	58	2	11
107	58	2	21
108	33	2	25 no bleeding[1]
109	58	3	20 no bleeding[2]

[1] Bleeding started 6 days after stopping injections of testosterone propionate.
[2] Dose of testosterone propionate reduced to 1 mg. daily and bleeding began 19 days later.

under the conditions set forth above. It was thought that such information would be useful as an indication of the dosage of androgen that probably would be required to precipitate bleeding in the presence of estrogen. This reasoning was based on previous observations that 1 mg. of progesterone daily was approximately the minimal amount that would inhibit estrogen-withdrawal bleeding under similar conditions (Hisaw and Greep, 1938), and at the same time was sufficient, when given in a single dose, to precipitate bleeding in the presence of estrogen. Accordingly, after the animals had received estrogen for 33 days or longer, testosterone or testosterone propionate was added to the treatment for a period of 5 days, the injections of estrogen being continued both during and after the administration of androgen. The daily dose of testosterone for the 5 days was either 10 or 20 mg., while that of testosterone propionate ranged from 1 to 9 mg. In a majority of the instances in which bleeding had not occurred within what was considered a reasonable time after an initial 5-day androgen treatment, a second was given at an increased dose while the injections of estrogen were continued without interruption.

RESULTS AND DISCUSSION

The data recorded in table 2 show that 4 monkeys receiving 10 mg. of testosterone daily for 5 days had not bled by the 9th to 12th

TABLE 2. PRECIPITATION OF BLEEDING IN THE PRESENCE OF ESTROGEN WITH TESTOSTERONE AND TESTOSTERONE PROPIONATE

Animal No.	Days of Pretreatment with 1000 I. U. Estrogen	Daily Dose Androgen for 5 Days, mg.	Results		Day Bleeding Started after 1mg. Progesterone
			Days	Bleeding	
98	33	10 (T) 30 (T)	12 30	no bleeding no bleeding	
100	33	10 (T) 30 (T)	12 10	no bleeding bleeding	
101[5]	33	10 (T)	9	no bleeding	
102[5]	33	10 (T)	9	no bleeding	
100	55[1]	1 5	33 17	no bleeding no bleeding	8, bleeding
103	49	5	17	no bleeding	
104	55[1]	1 5	33 17	no bleeding no bleeding	7, bleeding
105	55[1]	2 7	33 17	no bleeding no bleeding	9, bleeding
106	55[1]	2 7	33 17	no bleeding no bleeding	9, bleeding
107	55[1]	2 7	33 17	no bleeding no bleeding	9, bleeding
108	49	9	9	bleeding	
109	33[2]	3 9	33 8	no bleeding bleeding	
114	33[2]	5 9	33 33	no bleeding no bleeding	a. 17, no bleeding[4] b. 10, no bleeding[4] c. 8, no bleeding[4] d. 5, bleeding
141[5]	31	9 25[3]	20 26	no bleeding no bleeding	17, no bleeding 6, bleeding
142[5]	31	9 25[3]	20 26	no bleeding no bleeding	9, bleeding
143[5]	42	9	8	bleeding	
144[5]	42	9	8	bleeding	

(T) Testosterone. All others received testosterone propionate.
[1] Was given 500 I. U. of estrogen daily during first 37 days.
[2] Was given 500 I. U. of estrogen daily during first 15 days.
[3] 25 mg. of testosterone propionate given in one dose.
[4] a. 1 mg. of progesterone given; b. 1 mg. on two succeeding days; c. 2 mg. in one dose; d. 1 mg. daily for 3 days.
[5] Not castrated.

day thereafter, but of the 2 given a second treatment of 30 mg. daily for a like period 1 began bleeding on the 10th day, while the other

had not bled 30 days later. The observation period of 9 to 12 days following the treatments with 10 mg. may have been too brief, but the fact that 1 of the 2 animals receiving 30 mg. did not bleed within 30 days probably indicates that the smaller dose was considerably below that required for the precipitation of bleeding.

The greater androgenic action of testosterone propionate than testosterone suggested that of the two compounds the former probably would be the more effective for the precipitation of bleeding in the presence of estrogen. Consequently, the daily amounts of testosterone propionate used ranged from 1 to 9 mg. An examination of the data (table 2) will show that in no instance did bleeding follow the daily injection of 1 to 7 mg. of testosterone propionate for the 5-day period, but bleeding did occur on the 8th and 9th day in 4 of 7 animals given 9 mg. daily for a like period. These results, therefore, indicate that the minimal amount of testosterone propionate required to precipitate bleeding under the conditions of these experiments is about 9 mg. daily for 5 days.

A comparison of the effectiveness of testosterone propionate and progesterone in the precipitation of bleeding was made by testing the sensitivity of these animals to progesterone. Eight monkeys (100, 104, 105, 106, 107, 114, 141, and 142) that had not bled during an observation period of 17 to 33 days since the last 5-day period of testosterone propionate treatment were given a single dose of 1 mg. of progesterone subcutaneously in 0.25 cc. of sesame oil. Although the daily injections of estrogen were continued, 6 of these animals began bleeding 7 to 9 days later.

Two of the monkeys (114, 141) that failed to bleed following a 5-day treatment with 9 mg. of testosterone propionate daily also failed to menstruate following the injection of 1 mg. of progesterone. However, monkey 141 bled on the 6th day after a second injection of progesterone. Monkey 114, an adult and much larger than the others, did not bleed when 1 mg. of progesterone was given on two successive days, nor when 2 mg. was given in a single dose, but did menstruate on the 5th day after 3 daily injections of 1 mg. each. Age and size of the animal is probably of minor importance as a factor in this variation of response as monkeys comparable in these respects previously have been found to show individual differences in sensitivity to progesterone (Hisaw, 1942).

Testosterone propionate is apparently about 3 times as active as testosterone for the precipitation of bleeding in the presence of estrogen, while both of these androgens are much less effective than progesterone. The data (table 2) are such that an exact quantitative comparison cannot be made, but there are several general points of interest. A single dose of 1 mg. of progesterone is as effective as 150 mg. of testosterone or 45 mg. of testosterone propionate when distributed equally over 5 days. The minimal amount of progesterone

required to precipitate bleeding when given in 5 equal doses on 5 successive days was not determined, but it probably would be less than 1 mg. rather than more. In this connection it should be noted that 2 monkeys (141, 142) failed to bleed when given 25 mg. of testosterone propionate in a single injection, but when given 1 mg. of progesterone, 1 bled on the 9th day thereafter and the other on the 6th day following a second treatment.

A conspicuous difference between testosterone propionate and progesterone is also brought out when their ability to inhibit estrogen-withdrawal bleeding is compared with their effectiveness in precipitating bleeding in the presence of estrogen. Judging from the data presented here (table 1), 2 mg. of testosterone propionate seems to be about the minimal daily dose required to inhibit estrogen-withdrawal bleeding for a period of 20 days. Duncan, Allen and Hamilton (1941) in similar experiments on a larger series, succeeded in maintaining 3 monkeys for 25 days without bleeding by the injection of 1 mg. of testosterone propionate daily. It may be significant, however, that their priming treatment with estrogen was 500 I.U. for 10 days while ours was 1000 I.U. for 32 to 58 days.

Even so, it appears that 1 or 2 mg. of testosterone propionate daily is a sufficient dose to inhibit estrogen-withdrawal bleeding for 20 days or longer. In this respect the androgen compares favorably with progesterone which must also be given in daily doses of 1 mg. to produce this effect (Hisaw and Greep, 1938). Yet, this amount of progesterone (1 mg.), given in one injection, will precipitate bleeding in monkeys receiving 1000 I.U. of estrogen daily, while the amount of testosterone propionate required for this reaction is many times its effective dose for the inhibition of estrogen-withdrawal bleeding. One mg. of progesterone daily will also produce a progestational reaction in the endometrium, and less than half of this amount is equally effective if given simultaneously with estrogen (Hisaw, Greep and Fevold, 1937). In contrast with this, a daily dose of 10 or 25 mg. of testosterone propionate, either alone or in combination with estrogen, does not bring about noticeable progestational changes in the endometrium (Hartman, 1937; Engle and Smith, 1939) although this dosage is above that (9 mg.) required to precipitate bleeding in the presence of estrogen when given for 5 days.

The observations of Engle and Smith (1939), however, indicate that 25 mg. of testosterone propionate daily may have a weak progestational action on the endometrium. They found that the endometria of monkeys, which had been given 1 mg. of progesterone daily for 10 days followed by 25 mg. of testosterone propionate daily for an additional 10 days, showed progestational changes considerably greater in degree than could have been expected to be present at the conclusion of the treatment with progesterone. From this it would seem that testosterone propionate should be given in daily doses sev-

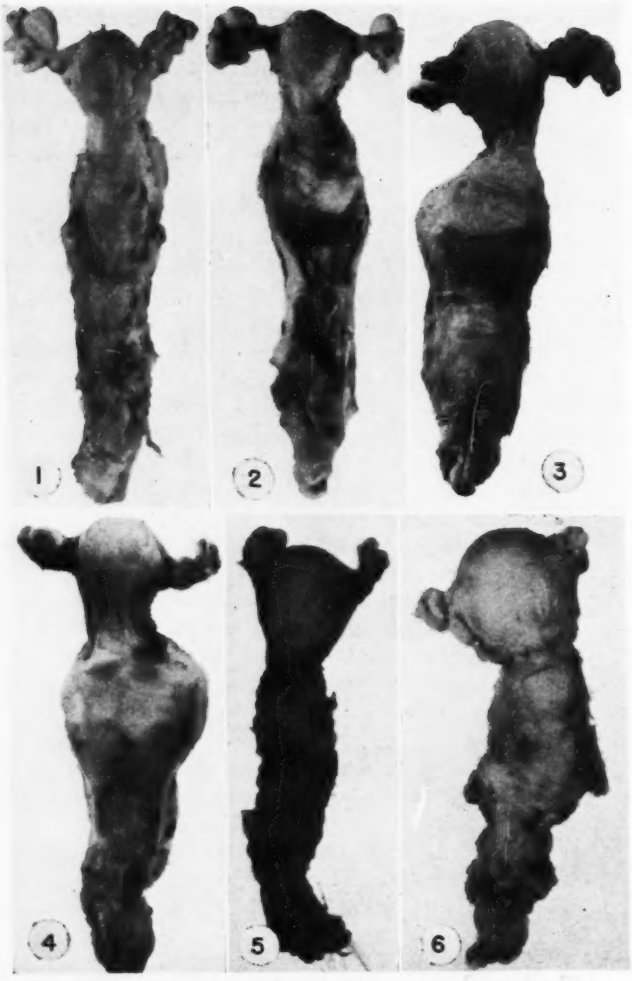

FIG. 1. Reproductive tract, monkey 101; received 1000 I.U. of estrogen daily for 47 days during which 10 mg. of testosterone was given daily for 5 days (34–38) without effect on bleeding (see table 1). Injections of estrogen were discontinued, and 10 mg. of testosterone propionate was given daily for the next 20 days. (×1).

FIG. 2. Reproductive tract, monkey 102; received same treatment as that given monkey 101 for the first 47 days, after which estrogen (1000 I.U. daily) and testosterone propionate (10 mg. daily) were given concurrently for 20 days. (×1).

FIG. 3. Reproductive tract, monkey 107; was given 1000 I.U. of estrogen daily for 46 days. The condition shown here is probably that present in the genital tracts of monkeys 101 and 102 (fig. 1 and 2) at the conclusion of the 47-day priming treatment with estrogen. (×1).

FIG. 4. Reproductive tract, monkey 108; was given 1000 I.U. of estrogen daily for 62 days. This probably represents the condition that would have been found in monkey

eral times greater than 25 mg. if a definite progestational reaction in the monkey is to be had. Evidence of a similar nature was also obtained from our own experiments. Treatment of 2 monkeys (101 and 102) that had been receiving 1000 I.U. of estrogen daily for 47 days, and had also received testosterone for 5 days (table 2) was continued as follows: in 1 animal (101) the injections of estrogen were discontinued, and 10 mg. of testosterone propionate was given daily for the following 20 days; while in the other (102) the estrogen was continued and the androgen given concurrently.

These 2 animals when killed at the end of the 20-day period of treatment showed no conspicuous differences in the gross appearance of the genital tracts (fig. 1 and 2). Nor were there any significant differences in the histological appearance of the endometria. The uterine glands of both contained considerable glycogen, but they were neither coiled nor dilated nor otherwise rsembled a progestational condition. This is quite significant with regard to uterine bleeding as it indicates that little if any histological modification was induced by the 5-day treatments with the testosterone propionate used to precipitate bleeding in the presence of estrogen, thus again (Hisaw, 1942) emphasizing the probability that a physiological conditioning of the endometrium is more important in rendering it refractory to estrogen than morphological modifications toward a progestational condition.

When the genital tracts of these two animals (fig. 1 and 2), which were given testosterone propionate, are compared with those of others given estrogen and progesterone, the progesterone-like action of the androgen becomes obvious. The structures most markedly affected are the cervix and vagina. The cervices and vaginae of 101 and 102 at the end of the priming period during which they received 1000 I.U. of estrogen daily for 47 days probably resembled the condition seen in 107 (fig. 3) which was given like treatment for 46 days. Also, had the androgen not been given during the last 20 days of the 67-day treatment of 102, its cervix and vagina probably would have resembled the condition seen in 108 (fig. 4) which was given 1000 I.U. for 62 days. This comparison shows clearly that the enlargement of the cervix and vagina induced by estrogen cannot be maintained by testosterone propionate (fig. 1) and is inhibited when the androgen and estrogen are given concurrently (fig. 2).

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102 (fig. 2) if testosterone propionate had not been added during the last 20 days of the 67-day treatment.

FIG. 5. Reproductive tract, monkey 72; received 1000 I.U. of estrogen daily for 21 days followed by 1 mg. of progesterone daily for 20 days. Compare with figure 1. (×1).

FIG. 6. Reproductive tract, monkey 69; received 1000 I.U. of estrogen daily for 21 days and continued to receive 3000 I.U. of estrogen and 1.0 mg. of progesterone daily for an additional 38 days. Compare with figure 2 and note similarity of gross appearance of vagina and cervix and difference in size of uterus. (×1).

Experiments in which progesterone was used yielded similar results. The gross appearance of the cervix and vagina of monkey 72 (fig. 5) which received 1000 I.U. of estrogen daily for 21 days, followed by 1 mg. of progesterone daily for 31 days, is comparable to that of 101 (fig. 1), while in like fashion these structures in 102 (fig. 2) are like those in monkey 69 (fig. 6) which was given 1000 I.U. of estrogen daily for 21 days and continued on a combination of 3000 I.U. of estrogen and 1 mg. of progesterone daily for an additional 38 days. Both testosterone propionate and progesterone suppressed the action of estrogen on the cervix and vagina, but it should be noticed that the uterus of monkey 69 (fig. 6) is much larger than that of monkey 102 (fig. 2) showing that the androgen, in the amounts given, unlike progesterone, did not act as a synergist with estrogen in promoting uterine growth.

Effects of the progesterone-like action of testosterone propionate are also seen in the vaginal mucosa and cervical glands. It has been found previously (Hisaw, Greep and Fevold, 1937) that progesterone can inhibit the cornification of the vaginal mucosa and metaplasia of the cervical glands which invariably occur during a prolonged treatment with estrogen. Testosterone propionate also has the same effect. The vaginal mucosa of monkey 102 was thin, there were very few mitoses, a Dierk's layer was absent, and there was only slight cornification in localized areas. This animal (102) had received 1000 I.U. of estrogen daily for 67 days during which a dose of 10 mg. of the androgen was given daily during the last 20 days This condition differed little if any from that seen in monkey 101 which received only testosterone propionate during the last 20 days of the 67-day period. Also, in neither of these animals were there metaplastic areas in the cervical glands. In sharp contrast with this, the cervical glands of both 107 and 108, which had received 1000 I.U. of estrogen daily for 46 and 62 days, respectively, showed extensive metaplasia, and the vagina in both was strongly cornified.

Testosterone propionate also had a progesterone-like effect on the sexual skin. The sexual skin of 3 young animals (142, 143, 144) underwent marked edematous development in response to estrogen during the priming period. When 9 mg. of testosterone propionate was injected daily for 5 days, the sexual skin of all 3 showed some loss of edema by the 5th day, after which deturgescence continued in the presence of estrogen after discontinuance of injection of the androgen. The deturgescence in these 3 animals, although pronounced, was neither as rapid nor as extensive as that following the injection of progesterone (Hisaw, 1942).

SUMMARY

These experiments furnish additional evidence that testosterone propionate has a weak progesterone-like action on the reproductive

tract of the castrated and intact immature female monkey. When given in adequate doses it will *a*) inhibit estrogen-withdrawal bleeding; *b*) precipitate bleeding in the presence of a maintenance dose of estrogen; *c*) inhibit estrogen in the enlargement of the cervix and metaplasia of the cervical glands; *d*) greatly decrease cornification of the vaginal mucosa; and *e*) bring about a loss of edema of the sexual skin.

The following quantitative comparisons with progesterone can be made: *a*) For the inhibition of estrogen-withdrawal bleeding for 20 days or longer, 1 mg. of progesterone daily is equivalent to about 2 mg. of testosterone propionate daily. *b*) For the precipitation of bleeding in the presence of 1000 I.U. of estrogen daily, one injection of 1 mg. of progesterone is as effective as 9 mg. of testosterone propionate daily for 5 days. *c*) For the production of progestational development of the endometrium, 1 mg. of progesterone daily is adequate, or less is required if given concurrently with estrogen, while 10 mg. of testosterone daily, either alone or in combination with estrogen, produces no noticeable histological modifications of the endometrium.

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VARIABLES AFFECTING THE BIOLOGICAL ASSAY OF ESTROGENS

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A NUMBER of modifications of the method of assay of estrogens using adult ovariectomized rats or mice have been described (Coward and Burn, 1927; Kahnt and Doisy, 1928; Marrian and Parkes, 1929; Allen, Dickens and Dodds, 1930; de Jongh, Laqueur and de Fremery, 1932; Marrian, 1933; Hain and Robson, 1936; D'Amour and Gustavson, 1936; Palmer and Dow, 1941), and a statistical study of the variables affecting the estimation of estrogenic activity using mice has recently been made by Emmens (1939a). The object of this investigation was to study some of the factors considered to affect the accuracy of the assay using rats so that a practical and convenient procedure with reasonable limits of error could be developed for checking the labeled potency of commercial estrogenic products distributed in an oily vehicle.

MATERIALS AND METHODS

Rats of Wistar strain inbred for several generations were ovariectomized at 3 to 4 months of age. Two or 3 days were allowed for the wounds to heal and the completeness of the ovariectomy was checked by vaginal smears after the operation. These control smears were made on a group of 500 rats for 10 days after the operation and 3 months later after estrone treatment for a similar control period. Since there was no evidence of incomplete ovariectomy or regeneration of ovarian tissue in this group, further control smears were considered unnecessary. The methods of taking and recording the smears were similar to those described by Kahnt and Doisy (1928) using the absence of leucocytes as the criterion of a positive response. The rats were assembled in groups of 15 to 20 on the basis of equal distribution of body weight in each group.

The dilutions of estrogens for the dosage levels were prepared from a stock solution of International Standard estrone (0.1 per cent) and estradiol monobenzoate (0.04 per cent) in 95 per cent ethanol by adding the required amount with a micro-pipette to a known weight or volume of corn oil. The dilutions were placed in a vacuum desiccator and the alcohol removed.

The usual practice was to begin the injections for a test on Monday taking the first smear 24 hours after the last injection. Subsequent smears were taken at intervals considered to be adequate in securing the maximum number of positive responses for the estrogen under test and consistent with practicability.

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The statistical methods described by Bliss (1935 a,b) for quantal response data and as applied by Morrell, Chapman and Allmark (1938) and Miller, Bliss and Braun (1939) to pharmacological assays have been used in calculating the regression lines, limits of error and evaluating the various modifications of the test outlined below. The equation numbers indicated in the text refer to the numbers given in Bliss (1935 a,b).

The position and slope of a series of regression lines. Data from 8 typical regression lines are presented in table 1. After the preliminary control smears the rats were primed with 1.5 μg. of estrone in 0.2 cc. of oil; this brought about stimulation of the cellular contents of the vagina in all the rats although none showed the absence of leucocytes. Two weeks following the

TABLE 1. POSITION AND SLOPE OF THE DOSAGE-RESPONSE CURVE AT FORTNIGHTLY INTERVALS USING LATIN SQUARE RANDOMISING, TWO INJECTIONS AND FOUR SMEARS

Curve No.	No. of Rats	Percentage Responses at Dosage Levels of				RD_{50} μg.	Slope b	X^2 for fit
		2.2 μg.	2.6 μg.	3.0 μg.	3.8 μg.			
1	66	5.9	25.0	50.0	76.5	3.03	9.34	0.438
2	66	31.2	41.2	64.7	75.0	2.73	5.26	0.470
3	66	37.5	64.7	64.7	87.5	2.42	5.68	0.723
4	65	37.5	56.2	50.0	88.2	2.57	5.68	1.965
5	65	37.5	43.7	81.2	93.7	2.50	8.45	1.549
6	65	25.0	35.3	43.7	81.2	2.93	6.48	0.925
7	65	43.8	75.0	64.7	87.5	2.21	4.73	1.955
8	64	33.3	50.0	62.5	88.2	2.60	6.69	0.263
					Mean	2.65	6.47	

priming dose they received two equally divided injections of estrone in oil, 0.2 cc. at each injection at an interval of 24 hours and smears were taken 24, 41, 48 and 65 hours after the last injection. Subsequent injections were made at fortnightly intervals using a 4×4 latin square method of randomising the groups.

The responses obtained at the different doses, the slope of the regression lines b, the RD_{50} (equation 27 after substituting $y = 5.0$) and the chi-square for the fit of the points to the provisional line (equation 7) are presented in table 1. It is seen that the RD_{50} (the dose producing 50 per cent response) varies without regularity from curve to curve. The chi-square for position (equation 19) was 21.78 which is above the probability value requisite for a constant position of the curves, chi-square = 14.06, p at 0.05 and n = 7 (Fisher and Yates, 1938). This indicates that at each fortnightly injection period there is a significant shift in the position of the regression line. On the other hand there is not a significant difference in the slope of the 8 curves shown in table 1 despite the variations in the value of *b* from 4.73 to 9.34. The value for chi-square b (equation 20a) is 3.91 which is well below the value of 14.06 with p at 0.05 and 7 degrees of freedom (Fisher and Yates, 1938).

The chi-square for the fit of the experimental points to each successive regression line is well below the value of 5.99 with p at 0.05 and n = 2, (Fisher and Yates, 1938), confirming the homogeneity of the group of rats. The effect of reversals of response (the higher dose producing a lower re-

sponse than the preceding lower dose) on the values of chi-square are shown in curves 4 and 7. The values for chi-square for the 8 lines were calculated from the fit of the points to the provisional line (fitted by inspection) and these values might have been reduced had the computed line been used.

OBSERVATIONS

Effect of Different Methods of Assembling Rats for Subsequent Injection on the Slope of the Dosage-Response Curve

Three groups of ovariectomized rats receiving the same priming, injection and smearing treatment as outlined above were assembled

TABLE 2. EFFECT OF DIFFERENT METHODS OF ASSEMBLING RATS FOR SUBSEQUENT INJECTION ON THE SLOPE OF THE DOSAGE-RESPONSE CURVE

Series	No. of Rats	No. of Curves	Slope b_e	Vb_e	X^2a	X^2b	Remarks
1	558	9	6.38	0.493	41.2	6.5	Same dose each time
2	479	8	5.05	0.619	15.5	2.8	Alternate high and lose dose
3	522	8	6.47	0.536	21.8	3.9	4×4 latin square

in different ways for subsequent injections at fortnightly intervals. Each of the four groups of rats in series 1 received the same dose each time, in series 2 the rats on the low dosage level were changed to the high dosage level for each successive curve, while the rats in series 3 were subjected to a 4×4 latin square method of randomising. The chi-square test $(b_{e1}-b_{e2})^2/Vb_{e1}+Vb_{e2}$ (Bliss, 1935b) shows that no significant difference exists between the three composite slopes recorded in table 2. The 4×4 latin square method of randomising is preferable since this ensures an orderly arrangement and permits more complete statistical treatment although the death of an occasional rat during the test period makes it difficult to employ the detailed statistical analyses.

The values of chi-square for position and slope in series 1 indicate that this method of assembling leads to greater variations than the other methods employed. However, the slopes as judged by the chi-square test (equation 20a) are the same within each series, but the position of the curves shifts showing that a standard is necessary for every assay.

Effect of the Number of Smears on the Slope of the Dosage-Response Curve

To determine if the number of smears recorded during the peaks of the estrous response influenced the slope of the dosage-response curve, the results obtained with 3 groups of rats receiving the same priming treatment as described above are shown in table 3. In series 1 the rats received the total dose in two injections at 9 A.M. Monday and

9 A.M. Tuesday and the curves calculated from the 5 smears taken at 4 and 11 P.M. Wednesday, 9 A.M. and 4 P.M. Thursday and 9 A.M. Friday. Curves were also calculated for series 1A on the basis of 4 smears omitting the smears made at the inconvenient hour of 11 P.M. Wednesday. In series 2 the rats received the total dose in two injections, one at 9 A.M. and one at 4 P.M. Monday and curves were calculated from the four smears taken at 4 P.M. Tuesday, 9 A.M. and 4 P.M. Wednesday and 9 A.M. Thursday. Similarly curves were calculated for series 2A using the same series with 3 smears, omitting the Thurs-

TABLE 3. EFFECT OF THE NUMBER OF SMEARS ON THE SLOPE OF THE DOSAGE-RESPONSE CURVE

Series	No. of Rats	No. of Curves	No. of Smears	b_e	Vb_e	RD_{50} μg.	X^2a	X^2b
1	559	9	5	7.15	0.452	2.40	25.6	7.1
1A	559	9	4	6.38	0.494	2.77	41.2	6.5
2	561	8	4	6.02	0.332	2.87	29.3	3.3
2A	561	8	3	5.20	0.301	3.26	22.6	2.8
3	510	8	1	4.66	0.321	2.83	15.5	3.9

day 9 A.M. smears. In series 3 the rats were injected at the same times as series 2 and smears were taken only at 2:30 P.M. Wednesday. As judged by the chi-square test there is a significant difference in slope between series 1 and 3 and 1 and 2A, but not between the slopes of the remaining curves.

From the results it is evident that the smears made at the inconvenient hour of 11 P.M. Wednesday for series 1 can be omitted without significantly altering the slope of the dosage-response curve and so the accuracy of the assay. Although quite consistent results as judged by the chi-square test can be obtained with two injections and only one smear (series 3), this method of injection and smearing gave the lowest value for b. Two divided injections in one day and 4 smears taken 24, 41, 48 and 65 hours after the last injection (series 2) appears to be a convenient and practical procedure because an assay can be completed in 4 days.

Effect of the Number of Injections on the Slope of the Dosage-Response Curve, the RD_{50} and the Limits of Error of the Assay

Six different groups of rats receiving the same preliminary treatment as described above received the dose of estrone in oil in one injection or divided among 2, 3, 4, 6 and 9 injections to measure the effect on the slope of the regression line, RD_{50} and limits of error of the test. It is seen (table 4) that increasing the number of injections from 1 to 6 results in a significant increase in the slope of the dosage-response curve and a definite reduction in the limits of error of the test. A preliminary experiment with 9 injections did not show evidence of

producing a steeper curve than that obtained with 4 injections. It is evident that increasing the slope of the curve has a greater effect in decreasing the limits of error of the test than increasing the number of animals. Increasing the number of injections beyond two did not significantly lower the RD_{50}, although there is some evidence of a pro-

TABLE 4. EFFECT OF THE NUMBER OF INJECTIONS ON THE SLOPE OF THE DOSAGE RESPONSE CURVE, RD_{50} AND LIMITS OF ERROR OF THE ASSAY USING ONE DOSAGE LEVEL OF STANDARD AND SAMPLE

Series[1]	No. of Injections	No. of Curves	No. of Rats	Slope b_c	Vb_c	RD_{50} $\mu g.$	Maximum Limits from True Potency as Per Cent, 95 Times in 100 with Responses Between 20 and 80% Using per Assay		
							40 rats	80 rats	160 rats
1	1	8	592	4.50	.462	3.73	64 to 157	73 to 138	79 to 126
2	2	8	551	6.66	.469	2.66	74 to 135	81 to 124	86 to 116
3	3	8	529	6.60	.348	2.79	74 to 135	81 to 124	86 to 116
4	4	14	896	8.48	.346	2.38	79 to 127	84 to 118	89 to 113
5	6	8	537	9.35	.453	2.34	81 to 124	86 to 116	90 to 111
6	9	4	232	8.15	1.005	2.24	78 to 128	84 to 119	88 to 113

[1] Series 1: Total dose in 1 inj. at 4 P.M. Monday, smears 24, 41, 48 and 65 hours after last injection.
Series 2: Two divided inj. at 9 A.M. and 4 P.M. Monday, smears as above.
Series 3: Three divided inj. 4 P.M. Monday, 9 A.M. and 4 P.M. Tuesday and smears as above.
Series 4: Four divided inj. 9 A.M. and 4 P.M. Monday, 9 A.M. and 4 P.M. Tuesday and smears as above.
Series 5: Six divided inj. 9 A.M., 12A.M., and 4 P.M. Monday and Tuesday and smears as above.
Series 6: Nine divided inj. 9 A.M., 12 A.M., and 4 P.M. Monday, Tuesday and Wednesday and smears as above.

gressive decrease in this value as the number of injections are increased.

Effect of Varying the Method of Giving the Injections on the Slope of the Dosage-Response Curve

The effect of constant concentration and of varying the concentration of the solution to be tested to suit the dosage level and the effect of giving the dose on the basis of the mean body weight of the whole group or the individual body weights are shown in table 5. From these results it is evident that there is no advantage to the accuracy of the assay gained by preparing separate solutions to suit the dosage levels, since there is not a significant difference in the slopes of the curves or RD_{50} of series 1 and 2.

To determine if the amount of estrone given to each rat should be based on the mean body weight of the group or on the individual body weight, two groups of rats were paired with respect to body weight and the dose of estrone given to series 3 on the basis of the mean body weight of the whole group while in series 4 the doses were varied to suit the individual body weight. The mean weight and standard deviation

TABLE 5. EFFECT OF VARYING THE METHOD OF GIVING THE INJECTIONS ON THE SLOPE OF THE DOSAGE-RESPONSE CURVE

Series	No. of Rats	No. of Curves	Slope b_e	Vb_e	RD_{50} μg.	Remarks
1	521	8	6.66	0.468	2.75	Concentration varied to suit dosage level, 2 divided injections 0.2 cc.
2	561	8	6.03	0.332	2.87	Constant concentration, 2 divided injections 0.2 cc. to 0.4 cc.
3	914	12	6.92	0.252	2.60	Conc. constant, 2 divided injections on basis of mean body weight 0.16 to 0.48 cc.
4	633	8	7.63	0.383	2.66	Conc. constant, 2 divided injections on basis of individual body weight 0.16 to 0.48 cc.

of each group was 216 ± 19.4 gm., respectively, at the beginning of the tests. The latter method of injection gave a steeper curve than the former, but the difference between the two composite curves is not significant.

For practical reasons in the preparation of the dosage levels the method of using one solution and two divided injections and increasing the amount to suit the dosage level regardless of body weight appears to be a satisfactory procedure.

Effect of Preliminary Priming, Interpriming, and the Time Interval Between Subsequent Doses on the Slope of the Dosage Response-Curve and RD_{50}

The effect on the slope of the dosage-response curve and RD_{50} of using rats without a priming dose of estrone and after a priming dose of estrone is shown in table 6. The results from the unprimed rats were obtained from 4 different groups in which each group received 4

TABLE 6. EFFECT OF PRELIMINARY PRIMING, INTERPRIMING AND THE TIME INTERVAL BETWEEN SUBSEQUENT DOSING ON THE SLOPE OF THE DOSAGE-RESPONSE CURVE AND RD_{50}

No. of Rats	No. of Curves	b_e	Vb_e	X^2a	X^2b	RD_{50} μg.	RD_{50} [1]S.D.	Remarks
286	4	6.06	.580	4.3	5.0	2.84		Dosed without priming
291	4	6.24	.835	2.4	4.8	2.68		Dosed after priming
382	7	5.95	.568	18.8	5.7	2.48		Interpriming
387	6	6.53	.600	9.6	1.0	2.58	0.205	Dosed at fortnightly intervals
485	8	6.60	.415	6.1	6.9	1.63	0.127	Dosed at weekly intervals
101	2	6.77	1.810	0.0	0.0	2.66		Dosed at fortnightly intervals

[1] Standard Deviation

dosage levels of estrone 5 days after ovariectomy. The rats were given 2 injections at an interval of 7 hours and smears were made 24, 41, 48 and 65 hours after the last injection. The results from the primed rats were obtained from 4 different groups of rats in which each rat received a priming dose of 1.5 μg. of estrone in 0.2 cc. of oil 10 days after ovariectomy and 2 weeks following the priming dose each group received 4 dosage levels of estrone. The injections and smearing times were the same as above.

It is quite evident that priming had a negligible effect on the RD_{50} and there is not a significant difference between the slopes of the com-

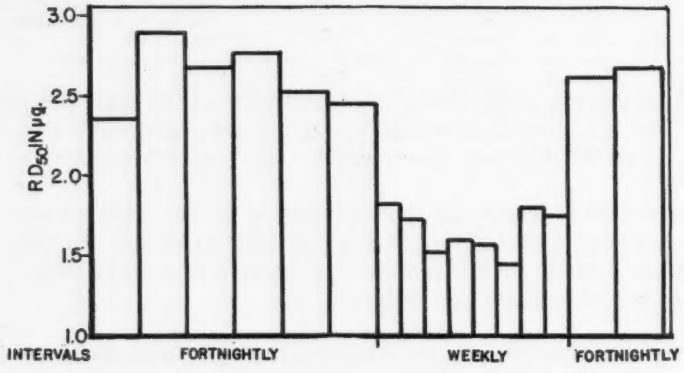

FIG. 1. Effect of fortnightly and weekly dosing on the RD_{50}.

bined regression lines. These results indicate that priming contributes very little if anything to the precision of an assay. There are too few curves in this experiment to evaluate adequately the values for chi-square for fit and chi-square for position since the results are not in agreement with those obtained with the larger series reported in this work.

The effect of interpriming was studied in another group of rats. These rats were primed with 0.2 cc. = 6 μg. of estrone, an amount adequate to produce complete squamous smears in the whole group. Two weeks after this priming dose a regression line was obtained using 4 dosage levels, 2 injections at an interval of 7 hours and smears made at 24, 41, 48 and 65 hours after the last injection. One week from the time they were injected they were given a second priming dose of 0.2 cc. = 6 μg. of estrone and 2 weeks following this a second regression line was obtained. This routine was continued until 7 regression lines were obtained and the results are shown in table 6. It is evident that the interpriming had very little effect in determining the position of the curve. The value of chi-square for position is 12.59 at $p = 0.05$ and $n = 6$ (Fisher and Yates, 1938). On the other hand, the

value of chi-square for slope is lower than this indicating that it is constant.

The effect of the time interval between tests was studied on another group of rats. The same rats were dosed for 6 successive times at fortnightly intervals, then dosed 8 times at weekly intervals and finally twice at fortnightly intervals (table 6). Although changing the time interval between subsequent dosing did not have any effect on the slope of the curve, it had a marked effect in reducing the RD_{50}. This reduction in the RD_{50} is ascribed to unabsorbed estrone exerting an effect on the subsequent doses at weekly intervals. There did not appear to be a continuous accumulation since the RD_{50} reached a common level as illustrated in figure 1. This cumulative effect is not observed with rats injected at fortnightly intervals since the RD_{50} of the unprimed rats is within the same range as for rats injected continuously at fortnightly intervals. The position and slopes of the regression lines in this series are constant both for the fortnightly and weekly treatment; chi-square = 11.07 p at 0.05, and n = 5 and chi-square = 14.06, p at 0.05 and n = 7.

Effect of the Amount of Oil Injected on the Slope of the Dosage Response Curve and RD_{50}

To determine if the amount of oil administered with each injection affected the slope of the dosage-response curve or the RD_{50}, a group of primed ovariectomized rats received 2 divided injections of estrone at an interval of 7 hours in amounts of 0.10, 0.12, 0.15, and 0.20 cc. at each injection for 2 successive experiments at fortnightly intervals. The amount of oil at each injection was then increased to 0.2, 0.25, 0.3, and 0.4 cc. for 6 successive tests and finally 5 more tests were completed using 0.1, 0.12, 0.15 and 0.20 cc. at each injection. The results are shown in table 7. It is seen that a relatively larger RD_{50}

TABLE 7. EFFECT OF THE AMOUNT OF OIL INJECTED ON THE SLOPE OF THE DOSAGE-RESPONSE CURVE AND RD_{50}

No. of Rats	Slope b	RD_{50} μg.	Amount of Oil
76	6.42	4.57	0.10 to 0.20 cc. at each injection
76	4.72	4.89	
76	5.43	2.88	0.20 to 0.40 cc. at each injection
76	7.16	3.31	
76	5.28	3.31	
76	4.00	3.23	
75	4.41	3.18	
73	5.07	2.68	
73	5.12	2.81	0.10 to 0.20 cc. at each injection
73	5.25	3.56	
73	7.76	3.08	
71	8.58	3.71	
68	4.50	3.91	

was obtained from the first two tests using the smaller amounts than with the 6 tests using the larger amounts of oil. However when the quantity of oil was reduced again the RD_{50} was within the same range as found for the larger amounts. It is evident, therefore, that the amount of oil injected within the limits shown here has not a significant effect on the slope of the dosage-response curve or RD_{50}.

Factors Influencing the Slope of the Dosage-Response Curve of Estradiol Monobenzoate

The following experiments were conducted with estradiol monobenzoate in order to confirm the work with estrone showing that

TABLE 8. FACTORS INFLUENCING THE SLOPE OF THE DOSAGE-RESPONSE CURVE FOR ESTRADIOL MONOBENZOATE

No. of Rats	No. of Curves	b_o	Vb_o	X^2a	X^2b	RD_{50} μg.	Remarks
868	13	6.83	0.183	41.9	8.7	.46	Concentration varied to suit the dosage level, two divided injections, 0.2 cc. primed rats
584	9	6.79	0.343	22.9	7.6	.56	Constant conc. two divided injections 0.2 to 0.4 cc. unprimed rats

when $n = 12$ p at $0.05x^2 = 21.02$
when $n = 8$ p at $0.05x^2 = 15.50$

dosing on the basis of increasing the amount of oil injected within certain limits gave approximately the same slope as injecting a constant amount of oil and increasing the concentration of hormone to suit the dosage level. In the first experiment 13 regression lines were obtained on a group of primed ovariectomized rats. These were given two divided injections of 0.2 cc. each at an interval of 24 hours using 4 dosage levels of the International Standard estradiol monobenzoate and smears were made 32, 48, 56, 72 and 80 hours after the last injection.

In the second experiment 9 regression lines were obtained from a group of unprimed ovariectomized rats. These were given two injections at an interval of 7 hours and smears were made 48, 65, 72 and 89 hours after the last injection. Four dosage levels were used and 0.2, 0.25, 0.3 and 0.4 cc. of an oil solution of the hormone were given at each injection.

The two treatments did not result in a significant difference in the slopes of the dosage-response curves (table 8). The position of the curves as judged by the chi-square test shows them to be variable in both cases while the slopes as shown by the chi-square test are constant within each series. In evaluating the activity of estradiol monobenzoate with estrone by comparing the RD_{50} of the values obtained

here with the RD_{50} of the rats treated with estrone (table 6), the ratio was found to be 5.98:1 and 5.13:1 in favor of the estradiol monobenzoate in the first and second groups, respectively.

Standard Curves for Estrone in Oil Using the Two and Four Equally Divided Injections

A relatively large number of regression lines were obtained using the same injection and smearing treatment on different groups of rats in the course of this work and in checking commercial samples of

TABLE 9. STANDARD CURVES FOR ESTRONE IN OIL USING THE TECHNICS OF TWO AND FOUR EQUALLY DIVIDED INJECTIONS

No. of Rats	No. of Curves	No. of Inject.	b_c	Vb_c	X^2a	X^2b	RD_{50} μg.	Remarks
1847	29	2	5.99	0.166	120.0	18.0	2.42	Two inj. in 24 hr. Int. Std.
681	14	2	5.95	0.386	—	7.4	—	Two inj. in 24 hr. samples
2556	38	2	6.01	0.078	166.6	23.7	2.83	Two inj. in 7 hr. Int. Std.
4403	67	2	6.00	0.053	300.0	43.1	2.65	Two inj. in 7 hr. and 2 inj. in 24 hr. combined Int. Std.
5084	81	2	6.00	0.047	—	50.5	—	Two inj. Int. Std. and samples comb.
2035	30	4	8.40	0.147	131.9	26.8	2.27	Four inj. in 48 hr. Int. Std.
475	8	4	7.73	0.535	—	3.6	—	Four inj. in 48 hr. samples
2510	38	4	8.25	0.115	—	33.1	—	Four inj. Int. Std. and samples comb.

Equation for two inj. $Y = 4.8234 + 6.0076\ (X - 1.3998)$
Equation for four inj. $Y = 5.1097 + 8.4000\ (X - 1.3696)$

estrone. It appeared worth while to combine these and calculate a composite slope with its variance and thus obtain a standard curve. The results are shown in table 9. The results of experiments using two equally divided injections at an interval of 24 hours and at an interval of 7 hours were calculated separately in order to show that there is not a significant difference in the slope of the combined regression lines using these two different technics. The combined slope of the regression lines obtained from the commercial samples was calculated separately since the position of the curve naturally shifts according to whether the samples were of labelled potency. Data from 81 lines utilizing 5084 rats receiving 2 equally divided injections were available. The slope of the composite curve was 6.00 with a variance of .047 and the chi-square value signifies that the slope of this curve is constant. Similarly the curve obtained by combining the 67 regression lines using two equally divided injections of the International Standard estrone was constant in slope but variable in position as shown by appropriate chi-square tests (table 9).

Thirty regression lines utilizing 2035 rats were available using 4 equally divided injections over a period of 48 hours and International Standard estrone. The position of this curve varies without regularity as indicated by chi-square while the slope is constant since chi-square $=42.55$ at 29 degrees of freedom and p at 0.05 (Fisher and Yates, 1938). Combining the regression lines from Standard and samples, 38 lines were obtained utilizing 2510 rats. This gave a composite slope of 8.25 with a variance of 0.115. This line is significantly steeper than the one obtained with two equally divided injections and consequently the limits of error of an assay can be considerably reduced by using 4 injections as indicated in table 4. The chi-square for position and slope (table 9) between groups of rats confirms the variability in position and constancy of slope found within groups of rats. Since there are no data available on the slope of the dosage-response curve for estrone for rats of other colonies it is not known to what extent the values found here can be used generally.

DISCUSSION

The slope of the dosage-response curve in quantal response assays serves as a measure of the change in effect per unit change in the logarithm of the dose, and the reciprocal of the slope is the standard deviation of the logarithm of the individual effective doses. The larger the value of b (the slope of the dosage-response curve) the smaller is the standard deviation of the individual effective doses and the accuracy of the test is increased accordingly. This relationship indicates the importance of using the slope as a criterion when comparing the precision of different assay methods as has been done throughout the work reported above. In a test of this kind the homogeneity of the animals is indicated by the goodness of fit of the results to a straight line when the logarithms of the doses are plotted against the corresponding responses after transforming the latter to probits. In this work 4 dosage levels were used to ensure at least 3 points for the line since zero response or 100 per cent response is not well defined in quantal response assays. In all the regression lines obtained in this work there was no indication, according to the chi-square test, that the experimental points did not fit a straight line.

Chi-square can be used to determine whether the position of a series of regression lines shifts significantly (Bliss, 1935b). A significant degree of movement of the regression line along the abscissa indicates a definite change in sensitivity of the colony of animals to the hormone (aperiodic variation) and since this change was shown to occur in the above experiments it is necessary to employ a standard of comparison with every test. Chi-square is also employed to reveal significant changes in slope of the regression lines or in other words changes in the extent of the individual variations to the same or

different treatments (Bliss, 1935b). By this means it was shown that the method of assembling rats for subsequent injection at fortnightly intervals was immaterial. On the other hand, the number of injections used (table 4) and the number of smears made at the peak of the estrus (table 3) are important factors in determining the slope of the dosage response curve. It was shown that a progressive increase in the slope of the dosage-response curve occurred when the number of injections by which the dose was given was increased from 1 to 6. Although 9 injections did not further increase the slope of the curve, it is possible that a further subdivision of the dose beyond 6 injections, at equally spaced time intervals, might produce the desired result.

The practical application of dividing the injections is the conservation of animals, since it is desirable to have b as large as possible consistent with economy of material and effort. It is seen (table 4) that an assay using 160 rats and 2 injections yields approximately the same limits of error as one using 80 rats and 6 injections. A further point of practical significance is brought out in the results shown in tables 5 and 8. It appears more practical to prepare one concentration of the estrogen and increase the volume of the injection to suit the dosage level.

Preliminary priming of rats and mice to make them more uniform in response has been recommended by D'Amour and Gustavson (1936) and Palmer (1939). According to the results obtained (table 6) priming did not give any significant advantage either in the homogeneity of the rats or on the slope of the dosage-response curve. Interpriming has also been recommended in mice by Palmer and Dow (1941) but in our work it did not increase the constancy of position or produce a more uniform response in the group of rats.

The time interval between doses is an important factor in determining the range of the RD_{50}. When rats are dosed at fortnightly intervals the RD_{50} is within the same limits as found for rats not previously injected, while weekly intervals between doses lower the RD_{50} considerably below the level obtained after the fortnightly intervals, figure 1.

The slope of the dosage-response curve for estradiol monobenzoate using 2 divided injections is within the same range as was found for estrone. The peak of the estrus is more prolonged with the esterified form and hence it requires a longer time to complete an assay. Although the relative activity of estrogens varies considerably with the species (Pedersen-Bjergaard, 1939), the ratio of the activity for estradiol monobenzoate to estrone found here is within the range reported by Curtis and Witt (1941).

The advantage of a standard curve and the simplification of the calculations through the use of curve numbers have been shown by Morrell, Chapman and Allmark (1938), Chapman and Morrell (1931,

1932). The composite slope, b_c, determined on a relatively large series has a smaller error than the b determined from individual tests. The values for slope reported here are for estrone and curve numbers derived from these values do not necessarily apply to estrogens other than estrone or to another colony of rats using a similar or different technic. Emmens (1939) reports the mean slope of 14 dosage-response curves utilizing 660 randomly mated mice to be 5.458 with a standard error of 0.543 using 2 injections over a period of 24 hours and a smearing procedure similar to the one employed here. Using the test $t=(M_1-M_2)/\sqrt{\sigma_1^2+\sigma_2^2}$ (Snedecor, 1937) this value is not significantly different from the value $b=6.0076$ found in this work ($t=0.625$). The slope of the dosage response curve for a pure line of mice, a derivative of Strong's CBA strain, has been reported by Emmens (1939b) to be less than that found for randomly mated albino mice.

Mice have not been found as adaptable as rats in this laboratory for the routine assay of estrogens (Pugsley, 1942). When the former animals were used continuously at fortnightly intervals, using a multiple injection treatment, considerable unabsorbed oil accumulated at the site of injection rendering the mice unsuitable for further use. There was some accumulation of oil at the site of injection in the rats, but it did not interfere with their use even when the same group was employed 15 to 18 times at fortnightly intervals.

SUMMARY

A standard is necessary in the assay of estrone in oil since the position of the dosage-response curve was found to shift within and between groups of rats. The slope of the curve is constant within and between groups of rats using the same smearing and injection methods.

The slope of the dosage-response curve was significantly increased and limits of error of an assay significantly decreased by increasing the number of injections from 1 to 6 equally divided injections.

Preliminary priming, interpriming and different methods of assembling rats for subsequent injection did not significantly influence the slope of the dosage-response curve.

The RD_{50} of rats injected at fortnightly intervals was within the same range as rats receiving the first injection, while rats injected at weekly intervals showed a definitely lower RD_{50}.

Within limits, the amount of oil administered at each injection is without a definite influence on the slope of the dosage-response curve or RD_{50} and the response to estrone does not appear to be related to body weight.

Using the same injection treatment, the slope of the dosage-response curve for estradiol monobenzoate was approximately the same as that found for estrone.

Using two equally divided injections the composite slope of the regression line for estrone utilizing 5000 rats was 6.00 with a standard error of 0.231 and the composite slope for 4 equally divided injections using 2500 rats was 8.25 with a standard error of 0.340.

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NEW BOOKS

CREATINE AND CREATININE METABOLISM. Howard H. Beard, Professor of Biological Chemistry, School of Medicine, Louisiana State University. 376 pages; 6 figures; 50 tables. $4.00. Cloth. Brooklyn, N. Y., Chemical Publishing Co., Inc. 1943.

SIXTEEN years have elapsed since the publication of Hunter's admirable monograph on creatine and creatinine. Since that time many advances have been made in the controversial field of creatine chemistry and for some years there has been a growing need for a monograph covering new and important work on the origin and metabolism of creatine and creatinine. An attempt to summarize and interpret the vast quantity of data which has accumulated in this field since 1926 has been made by Howard H. Beard in a new monograph entitled "Creatine and Creatinine Metabolism."

The origin and metabolism of creatine and its dehydration product, creatinine, have been the subjects of vigorous debate in the chemical literature since the discovery of creatine by Chevreul in 1885. However, Hunter was able to effect some order from the chaos engendered by hundreds of publications appearing in this field before 1926, and to establish a certain degree of continuity and harmony among the many divergent views contained in these reports. In the present monograph an attempt is made to reopen many ancient arguments concerning the metabolism of creatine and creatinine. In the opinion of the reviewer certain of these arguments might well have been permitted to lie dormant until further exploration of this difficult field has been made by means of isotopes or by new techniques of like specificity.

The book is controversial throughout. Indeed, much of it is argumentative to the point of being frankly polemical. The problem of the origin of creatine, for example, which in the opinion of many biochemists was well advanced toward a satisfactory solution by the brilliant studies of Schonheimer, duVigneaud, Borsook and their respective collaborators, has been reopened and the significance of many of their results questioned. For the most part, only the author's work is cited as being in conflict with the results of these studies, and little attention is given to the great volume of work which was substantiated by the modern studies with isotopes. The work of Schonheimer, duVigneaud and others, who showed by means of isotopes that creatine was formed from glycine, arginine and methionine, is admitted by Beard as being beyond criticism. He maintains, however, that the negative evidence secured by these workers for the participation of other amino acids cannot be taken seriously, and clings resolutely to his former theory that almost unlimited creatine production may occur from widely different materials.

The theory proposed by Beard and collaborators that "all amino acids of the protein molecule studied have the power of increasing the normal creatine content of young rat muscle" has been vigorously attacked by Fisher and Wilhelmi. These authors admit that Beard's conclusion is consistent with his experimental evidence, but contend nevertheless, that there is no proof whatsoever of its validity. They state further that the increase in muscle creatine which Beard obtained from the administration of a variety of substances can be explained in part by dehydration arising as a consequence of the high concentration of unabsorbed amino acids in the gut; and that finally if creatine synthesis and creatine deposition did occur it need not mean that any part of the agent responsible for the synthesis was incorporated into the creatine molecule itself. "That is, an increase in muscle creatine

concentration is not itself an indication that the agent producing the increase is a source of the extra creatine." The statement that dehydration may have been responsible for the increase in creatine which Beard found in the muscles of his animals is challenged by him with the remark: "How could dehydration of the organs occur if the amino acids were not absorbed?" If this comment was meant to be taken seriously it points to a regrettable lack of familiarity on the part of the author with the physiology of dehydration, since it is well known that many pharmacological agents produce dehydration of the body by virtue of their *failure* to be absorbed.

In appraising the results of studies on the origin of creatine which Beard has published in the past several years, and to which he makes repeated reference in his monograph, it is well to keep in mind another statement by Fisher and Wilhelmi, namely, "it is clear that increase in rate of creatine excretion is not an indication of increase in rate of creatine synthesis from the agent inducing the creatinuria." The fact that creatinuria may be induced by a variety of non-specific measures such as the administration of selenium or phosphorus, or even sodium chloride, may be offered in support of this conclusion. The only possible justification at the moment for relying on the degree of increase in creatine excretion as a measure of the extent to which a given substance has been incorporated into the creatine molecule would be in those instances in which the materials responsible for the increased excretion have been shown by means of isotopes to be precursors of creatine.

The author pays repeated tribute to Miller and Dubos for their discovery of the "creatinine" organism. He refers repeatedly to the bacterium as an "enzyme" and reports great success in using it to achieve greater specificity with the Jaffe reaction. Without meaning to detract from the usefulness of the Miller and Dubos reaction, it should be pointed out that since the intact bacillus is used in this reaction for the selective destruction of creatinine the term "enzyme" cannot be properly applied. Although destruction of creatinine may be accomplished by an enzyme system present in the organism, the term "enzyme" itself is generally reserved for a cell-free suspension which has been at least partially purified.

The physiological relationship between creatine and creatinine has been a subject of controversy for many years. The observation that feeding creatine is followed by its partial or complete disappearance without detectable influence on the output of creatinine was believed at one time to show that despite the similarity in structure between these substances, they should be regarded as being independent of each other in metabolism. Much of the difficulty inherent in this problem was removed by the studies of Schonheimer who showed that when creatine containing N^{15} was fed, creatinine containing N^{15} appeared in the urine and in amount equal to that of the isotopic creatine which had disappeared from the tissues.

In taking issue with Schonheimer's negative results for the reverse reaction, *i.e.*, the conversion of creatinine to creatine, Beard seems to contradict himself. For example, on page 131, in the chapter on the biological relationship between administered creatine and creatinine, the author states: "Recently Bloch and Schonheimer fed creatine containing N^{15} to rats. Creatinine containing N^{15} was isolated from the urine. This is direct proof that creatine is changed into creatinine. . . ." On page 133, however, the author goes on to say: " . . . nevertheless their negative results with creatinine are open to serious question since we have shown with over 80 young rats that the creatine-creatinine transformation does not occur while the reverse process did occur."

Beard's statement that "If it were true that creatine is transformed into creatinine in the body then an increase in creatinine excretion should be accompanied by an increase in creatine formation" is not biologically sound. This reasoning would imply that no regulatory mechanism for the conversion of creatine to

creatinine exists. The constancy of creatinine excretion under conditions of marked fluctuation in the levels of concentration and excretion of creatine argues for a carefully controlled regulation of this conversion.

The author's statements in refutation of the old distinction which was formerly made between exogenous and endogenous metabolism of creatine appear convincing. His claim that he was the first to offer evidence against this differentiation may well be left for others to decide. It might be mentioned, in this connection, that it is to the experiments of Schonheimer and his group to which most biochemists turn for evidence against the distinction which was once made between endogenous and exogenous metabolism.

Chapter XIII attempts to cover the conflicting literature on creatine-creatinine metabolism and the hormones by means of an extensive outline. Most readers not thoroughly familiar with this large and controversial field will be confused by the long list which the author has made from reports in the literature of the positive and negative effects of various hormones on the metabolism of creatine and creatinine.

In the chapter entitled "Fuel of Muscular Exercise," etc., the author has launched a vigorous attack against the evidence secured by Hellebrant, King, Maison, Horvath and others for the lack of effect of glycine and gelatin on the output of human energy. The failure of these workers to secure positive results is explained by Beard as follows: *a*) Not all subjects were fed glycine or gelatin in amounts sufficient to elicit a response; *b*) some subjects were on diets low in protein beforehand and as a consequence the glycine may have been utilized for protein synthesis; and *c*) "normal physiological variations between the individuals tested" very likely accounted for some of the results. The validity of the author's explanations for the failures reported by these workers to obtain an increase in the output of energy from glycine and gelatin must be left for future workers to decide. Arguments of the following type, however, cannot be admitted as scientific or as lending weight to the author's contention: "Another criticism of gelatin feeding experiments is that the observers always take for granted that the gelatin is completely digested and absorbed. This may not always be the case, and cases of muscular dystrophy have been cured by simply adding a pancreatic digest to the food ingested." A survey of the medical literature of the past decade has not revealed one clearly authenticated instance of a cure of muscular dystrophy in a human subject by any means whatsoever. Again, in attacking the negative evidence for the effect of glycine on the energy output, the author says of King's work: "If the protein of the diet is capable of furnishing the glycine requirements of the body then why is it that many thousands of individuals suffer from weakness and fatigue 'myasthenia mitis' and why are they able to increase their resistance to this weakness and fatigue when glycine is ingested? This fact is well known to the medical profession." That this *is* a fact or that it is admitted by the medical profession may be challenged vigorously on the basis of recent negative reports in the medical literature. Further conclusions made by the author in regard to the metabolism of glycine in the myopathies likewise seem to the reviewer to rest on exceedingly weak premises. The implication is frequently made that glycine and gelatin may be regarded as specific in the treatment of the myopathies, notwithstanding the fact that modern medical literature abounds with evidence to the contrary. Even the original proponents of the glycine and gelatin treatment for the myopathies no longer make claims for its efficacy. At least no papers purporting to answer the negative results reported by an impressive list of investigators have appeared during the last several years. Although the author may be personally convinced that glycine and gelatin are effective in the treatment of these diseases a word regarding the many negative reports of their value would seem to be in order.

In the final chapter on the "Relation of Amino Acids, Amines and Guanidine

Bases to Tumor Growth and Regression" the author throws scientific discretion to the winds. In speaking of substances which prevent proliferation of cancer cells he states: "Since the cancer is a parasite it robs the body of these essential constituents" (viz., amino acids, proteins, nucleoproteins, vitamins, enzymes and hormones) "and utilizes them to promote its own growth. This creates a deficiency or imbalance of these substances with the result that the cancer tissue absorbs most of the constituents of the diet as well as of the animal tissues, cachexia intervenes and the emaciated animal finally dies." At best this can be accepted only as a hypothesis subject to proof or rejection by experiment. Regarding his aqueous extract of the suprarenal cortex of sheep, Beard goes on to say: "From 10–15 per cent of the Walker sarcomas disappeared in our rats injected with this extract. Even before our experimental work was started this same extract had been shown to cause 50 out of 100 patients (from a total of over 7500 suffering from hopeless malignancies) to completely lose their tumors and live much longer than the usual 5 years which would have been allowed them by cancerologists (Coffey and Humber)." It would appear that the author takes the work of Coffey and Humber very seriously, and has not taken the trouble to examine the modern cancer literature which abounds in evidence of a contrary nature. Unfortunately there is little justification for the emphasis which the author gives to such claims and it would seem that they could only have been accepted without due regard to all aspects of the question and an almost complete neglect of the obvious methods open for their confirmation or rejection.

CHARLES L. HOAGLAND

VITAMINS AND HORMONES. Vol. 1. Edited by Robert S. Harris, Associate Professor of Nutritional Biology, Massachusetts Institute of Technology, and Kenneth V. Thimann, Associate Professor of Plant Physiology, Harvard University. New York, Academic Press, Inc. $6.50. xvii and 452 pp. 1943.

THE present volume is the first of what is hoped to be a succession of yearly chronicles of progress in the fields delimited by the title. The Editors themselves have stated the scope of the series and the responsibility of the contributors: "To accumulate, correlate and digest the current literature in a field in which research is active and to point out where knowledge is incomplete requires a thorough grasp of the subject. To indicate the directions in which future research would be most fruitful and useful one must have sound imagination. Each chapter in this volume was written by a well-qualified investigator who endeavored to evaluate the present status of his special subject and to indicate what knowledge is lacking." How well the Editors and contributors together contrived to justify these high ideals will be apparent to the readers of the several chapters presented in this symposium.

A wide range of subject material is covered in the ten chapters of the book, ranging from an excellent discussion of choline and its dietary significance through accounts of the relationships of vitamins to amino acids, of the photoreceptor functions of vitamin A and the physiology of anti-pernicious anemia material to end finally with reviews of the present status of the steroid hormones. These and several other chapters constitute critical reviews of their various subjects, and are, in the main, satisfactory as to provocativeness and adequate in critique. It is, of course, inevitable that in such a symposium, certain subjects will be dealt with less expertly than others. For example, the chapter on the appraisal of nutritional status struck the reviewer as placing more emphasis on authority and being more didactic in tone than was the general rule for the remainder of the volume. Nevertheless, in this age of specialization, one should count himself lucky to find even a a fraction of such a series of review articles acceptable to his tastes and pertinent to his interests.

The union of vitamins and hormones into a single field, as indicated in the title, is the result of no accidental design. Biologists have long been tantalized by the many similarities and the equally intriguing lack of relationships between the two classes of substances. Consider, for example, that growth may be controlled by experimental manipulation of either vitamin A or the growth hormone, or that, within limits, vitamin D and parathormone may be used interchangeably in the regulation of blood calcium concentration. On the other hand, relatively little evidence has been forthcoming in support of the view that symptoms of the deficiency diseases are brought about by a failure of formation or utilization of hormones.

Considerable progress has attended studies on the energetics of vitamins, with the result that today there is a well-marked tendency to regard vitamins as component parts of enzyme systems which, through some caprice of evolution, are not capable of synthesis in the mammalian organism. Indeed, this point of view is stated in its most advanced form in the chapter by Williams on the significance of vitamins in tissues, where it is suggested that the vitamins act as "promoters," that is, as substances which accelerate catalytic reactions.

The part played by the hormones in the reactions involving energy transformations is less clear than is the case with the vitamins. In general, the hormonal reactions can be demonstrated only in animal tissues. This concept concerning vitamin and hormone action was stated explicitly to the reviewer in the suggestion by a well-known biochemist that perhaps the fundamental difference between the two substances lay in the facts that the vitamin reactions could be carried out in the test-tube while the hormonally controlled ones required the presence of intact cells. It may well be that this difference will find explanation in the traditional use of anatomical methods in endocrinology, but in any case the fact remains that the hormonal activities have not yet been ripped from the concealment of the cellular envelope. However, the systematic study of enzymatic reactions has recently begun to yield significant information concerning the hormones. The suggestion that the destruction of steroid hormones in the liver may be under enzymatic control, and the discovery that thyroid hormone formation may be inhibited in the animal by drugs which are well known antienzymes, point the way toward a better understanding of the interrelationships between the various elements which participate in the chemical physiology of the animal organism.

The breadth and amount of the material encompassed by the book, and above all its emphasis on further and more critical investigation of the tentative conclusions make it a necessary addition to reference works available to research workers in the fields of nutrition and endocrinology alike. It is to be hoped that the succeeding volumes will maintain the high standards set by the first.

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